09/623307
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Luis A. Cruz
Printed name of person mailing correspondence

Signature of person mailing correspondence

Substitute Form PTO 1390 U.S. Department of Commerce Patent and Trademark Office TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	Attorney's Docket Number: 50026/024001 U.S. Application Number: Not Assigned Yet

INTERNATIONAL APPLICATION NUMBER	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
JP 10/48187 PCT/JP99/00954	~~February 27, 1998~~ February 26, 1999	February 27, 1998

TITLE OF INVENTION:	COMPOSITION FOR TRANSPORTING NEGATIVELY CHARGED SUBSTANCES
APPLICANTS FOR DO/EO/US:	Naoto Oku, Mamoru Nango, Hideki Miyazaki, and Hiroyuki Sakakibara

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1.	X This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2.	□ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3.	X This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4.	X A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5.	A copy of the International Application as filed (35 U.S.C. 371(c)(2)). X a. is transmitted herewith (required only if not transmitted by the International Bureau). □ b. has been transmitted by the International Bureau. □ c. Is not required, as the application was filed with the United States Receiving Office (RO/US).
6.	X A translation of the International Application into English (35 U.S.C. 371(c)(2).
7.	□ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)). □ a. are transmitted herewith (required only if not transmitted by the International Bureau). □ b. have been transmitted by the International Bureau. □ c. have not been made; however, the time limit for making such amendments has NOT expired. □ d. have not been made and will not be made.
8.	□ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9.	□ An oath or declaration of the inventors (35 U.S.C. 371(c)(4)).
10.	□ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5).
11.	□ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12.	□ An assignment for recording. A separate cover sheet in compliance with 37 3.28 and 3.31 is included.

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13.	☒ A FIRST preliminary amendment. ☐ A SECOND or SUBSEQUENT preliminary amendment.
14.	☐ A substitute specification.
15.	☐ A change of power of attorney and/or address letter.
16.	☐ Other items or information:

17.	☒ The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492(A)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international serach fee (37 CFR 1.455(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $ 970.00 ☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $ 840.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $ 690.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1) - (4) $ 670.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $ 96.00		

			ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00	
Surcharge of $130 for furnishing the oath or declaration later than ☐ 20 OR ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$ 0.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	25 - 20 = 5		5 x $18.00	$ 90.00	
Independent claims	2 - 3 = 0		0 x $78.00	$ 0.00	
Multiple dependent claims (if applicable)			+ $260.00	$ 0.00	
			TOTAL OF ABOVE CALCULATIONS =	$ 930.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed with this request (Note 37 CFR 1.9, 1.27, 1.28).				$ 465.00	
			SUBTOTAL =	$ 1395.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 OR ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$ 0.00	
			TOTAL NATIONAL FEE =	$ 1395.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property.			+	$ 0.00	
			TOTAL FEES ENCLOSED =	$ 1395 00	
				Amount to be refunded	$ 0.00
				charged	$ 0.00

☒ a. A check in the amount of $ 1395.00 to cover the above fees is enclosed.
☐ b. Please charge my Deposit Account No. 03-2095 in the amount of $ [**.**] to cover the above fees.
☒ c. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 03-2095.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Signature
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Reg No. 30,162

ATTORNEY DOCKET NO. 50026/024001

Applicant or Patentee	: NAOTO OKU et al.
Serial or Patent No.	: 09/623,307
Filed or Issued	: August 28, 2000
Title	: COMPOSITION FOR TRANSPORTING NEGATIVELY CHARGED SUBSTANCES

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

[ ] the owner of the small business concern identified below:
[X] an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Small Business Concern: DNAVEC RESEARCH, INC.

Address of Small Business Concern: 25-11, KANNONDAI 1-CHOME, TSUKUBA-SHI, IBARAKI 305-0856, JAPAN

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled COMPOSITION FOR TRANSPORTING NEGATIVELY CHARGED SUBSTANCES by inventors NAOTO OKU, MAMORU NANGO, HIDEKI MIYAZAKI, and HIROYUKI SAKAKIBARA described in

[ ] the specification filed herewith.
[X] application serial no. 09/623,307, filed August 28, 2000.
[ ] patent no. _____, issued _____.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e). NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Assignee Name:

Assignee Address:

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent on which this verified statement is directed.

Name: Hirotaka Nakatomi

Title: President

Address: DNAVEC RESEARCH, INC. 25-11, KANNONDAI 1-CHOME, TSUKUBA-SHI, IBARAKI 305-0856, JAPAN

Signature: _____ **Date:** 26 Dec, 2001

8-29-00

Patent
Attorney Docket No. 50026/024001

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Luis A. Cruz
Printed name of person mailing correspondence

Signature of person mailing correspondence

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Naoto Oku et al. Art Unit:

Serial No.: Not Assigned Yet Examiner:

Filed: Herewith

Title: COMPOSITION FOR TRANSPORTING NEGATIVELY CHARGED SUBSTANCES

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to examining this application, kindly amend the claims as follows:

In the Claims:

Cancel claims 1-25.

Please add the claims as follows:

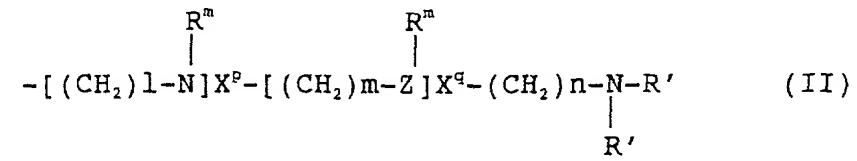
26. A composition comprising a polyealkylenimine having two or more hydrophobic groups or its salt.

27. The composition of Claim26, wherein the hydrophobic group is a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid residue.

28. The composition of Claim 26, wherein the polyalkylenimine having two or more hydrophobic groups is a compound represented by formula (I):

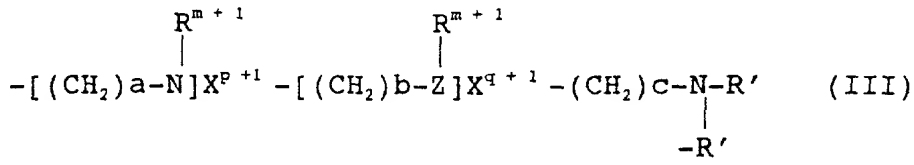
$$R'-\underset{\displaystyle R'}{\underset{|}{N}}-(CH_2)p-[\underset{\displaystyle R^1}{\underset{|}{N}}-(CH_2)q]X^m-[\underset{\displaystyle R^1}{\underset{|}{Z}}-(CH_2)r]X^n-\underset{\displaystyle R'}{\underset{|}{N}}-R' \qquad (I)$$

wherein the base skeleton may contain an amide bond; Z represents a carbon or nitrogen atom; R′ represents hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid residue; two R′s binding to the same nitrogen atom can be identical or different; a side chain R_1 is hydrogen, a cholesterol residue, saturated or unsaturated alkyl group, saturated or unsaturated acyl group, or saturated or unsaturated acyloxycarbonyl group, phospholipid residue, or below formula (II); and p, q, r, X^n, X^m represent arbitrary natural numbers:



(II)

wherein the base skeleton and the side chain R^m may contain an amide bond; Z represents a carbon or nitrogen atom; R′ represents hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid residue; two R′s binding to the same nitrogen atom can be identical or different; R^m is hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, or a saturated or unsaturated acyloxycarbonyl group, a phospholipid residue, or below formula (III); and l, m, n, X^p, X^q represent arbitrary natural numbers:



(III)

wherein the base skeleton and the base skeleton of the side chain R^{m+1} may contain an amide bond; Z represents a carbon or nitrogen atom; R′ represents hydrogen, a cholesterol residue, a saturated

or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid residue; two R's binding to the same nitrogen atom can be identical or different; and a, b, c, X^{p+1}, X^{q+1} represent arbitrary natural numbers.

29. The composition of Claim 26, comprising the repeating structure of formula (IV) in the base skeleton:

$$- CH_2 - CH_2 - NH - \qquad (IV).$$

30. The compositions of Claim 29, wherein two to five molecules of tetraethylenepentamine are linked in a linear manner.

31. The composition of Claim 30, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups.

32. The composition of Claim 30, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of a butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl groups.

33. The composition of Claim 29, wherein the structure containing the formula (IV) are linked in a branched manner.

34. The composition of Claim 33, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups.

35. The composition of Claim 33, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl groups.

36. The composition of Claim 26, wherein the base skeleton contains a spermine structure.

37. The composition of Claim 36, wherein two to five molecules of spermines are linked in a linear manner.

38. The composition of Claim 36, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups.

39. The composition of Claim 36, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of a butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl groups.

40. The composition of Claim 36, wherein the spermine structure is linked in a branched manner.

41. The composition of Claim 39, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups.

42. The composition of Claim 39, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl groups.

43. The composition of Claim 26, further comprising phospholipid.

44. The composition of Claim 43, wherein the phospholipid is neutral or basic phospholipid.

45. The composition of Claim 44, wherein the phospholipid comprises phosphatidylethanolamine, or phosphatidylcholine skeleton.

46. The composition of Claim 45, wherein the phospholipid is dioleylphosphotidylethanolamine, or phosphotidylcholine.

47. A complex comprising a physiologically active substance comprising a negative charge and a composition of Claim 26.

48. The complex of Claim 47, wherein the physiologically active substance comprising a negative charge is a nucleic acid or its derivative.

49. A method for introducing a physiologically active substance comprising a negative charge to cells, said method comprising a step of contacting the complex of Claim 47 with cells.

50. A kit for preparing the composition of Claim 44, comprising phospholipid and a polyalkylenimine having two or more hydrophobic groups per molecule or its salt.

REMARKS

The present amendment conforms the claims of this application to U.S. practice.

If there are any charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: August 28, 2000

Susan M. Michaud

Paul T. Clark — Susan M. Michaud
Reg. No. 30,162 — Reg. No. 42,885

Clark & Elbing LLP
176 Federal Street
Boston, MA 02110
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ATTORNEY DOCKET NO. **50026/024001**

Applicant or Patentee : NAOTO OKU et al.
Serial or Patent No. :
Filed or Issued : August 28, 2000
Title : COMPOSITION FOR TRANSPORTING NEGATIVELY CHARGED SUBSTANCES

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c)) - SMALL BUSINESS CONCERN

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[X] an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Small Business Concern: DNAVEC RESEARCH, INC.

Address of Small Business Concern: 25-11, KANNONDAI 1-CHOME, TSUKUBA-SHI, IBARAKI 305-0856, JAPAN

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I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled COMPOSITION FOR TRANSPORTING NEGATIVELY CHARGED SUBSTANCES by inventors NAOTO OKU, MAMORU NANGO, HIDEKI MIYAZAKI, and HIROYUKI SAKAKIBARA described in

[X] the specification filed herewith.
[ ] application serial no. _____, filed August 28, 2000.
[ ] patent no. _____, issued _____.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Assignee Name:

Assignee Address:

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent on which this verified statement is directed.

Name: _____

Title: _____

Address: DNAVEC RESEARCH, INC. 25-11, KANNONDAI 1-CHOME, TSUKUBA-SHI, IBARAKI 305-0856, JAPAN

Signature: _____ **Date:** _____

16/pts.

SPECIFICATION

COMPOSITION FOR TRANSPORTING NEGATIVELY CHARGED SUBSTANCES

Technical Field

The present invention relates to compositions for transporting negatively charged substances into cells.

Background Art

In chemotherapy, a system for transporting a target drug into target cells or intracellular tissues, namely the Drug Delivery System (referred to as "DDS" hereafter), is an important technology. Needless to say, the introduction of a gene into desired cells is a pivotal technology in gene therapy as well. There are two principal methods for introducing genes into cells.

One is the method using a viral vector. This method includes a method for introducing a desired exogenous gene on the genome of a virus into cells by infecting with the virus. The other is the method in which a desired gene or a vector containing said gene is sealed-in or carried in an artificial or a semi-artificial carrier. This method comprises transporting substances into the desired organ (a target organ), cell (a target cell), or organelle (a target organelle) by letting each process relating to the behavior and transport of the subject *in vivo* depend on the physiochemical properties of the carrier itself. Carriers used in this method are, for example, liposomes (F. Ledley et al., Human Gene Therapy 6, 1129-1144, 1995), proteins (Human Gene Therapy 5, 429, 1994), peptides (Proceedings of National Academy of Sciences of United States of America 90, 893, 1993), synthetic macromolecular compounds (Tang et al., Human Gene Therapy 4, 823-832, 1997), and (reconstituted) Sendai viruses (Exp. Cell Res. 159, 399, 1985), etc.

The method of using a liposome as a carrier has been contrived in many ways. Recently, based on the fact that a DNA molecule is a polyanion, the introduction of a DNA into a target cell was attempted by using, as a liposome, a cationic lipid that

has a static affinity to the DNA molecule, and which can easily form a complex. As such cationic lipids, lipofectin, 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP), 1,2-dimyristyloxypropyl-3-dimethylammonium bromide (DMRIE) (F. Ledley et al., Human Gene Therapy 6, 1129-1144, 1995), transfectum, etc., are known. However, there was a problem since a cationic lipid is easily decomposed by lysosomes, and such, after been taken up into cells through the endocytosis process and thus lowering the gene transfer efficiency.

To solve these problems, the use of cationic macromolecules has been attempted. For example, Kim et al. disclosed a gene introduction system in which polylysine is bound to a hydrophobic group, introduced into a plasma protein by hydrophobic interaction and statically bound to a DNA molecule (U.S. Patent No. 5,679,559). Zhou et al. prepared a liposome consisting of a polylysine into which a hydrophobic group was introduced, and a phospholipid (X. Zhou et al., Biochemica et Biophysica Acta. 1065, 8-14, 1991, X. Zhou et al., ibid. 1189, 195-203, 1994). Zhou et al. have successfully lowered cell damage by using poly-L-lysine and improved affinity of the DNA/liposome complex to a target cell by introducing lipid residues. However, in order to introduce the liposome into cells efficiently, the cells had to be processed with chemicals in advance. Also, WO97/45442 discloses a liposome including polyamine into which a cholesterol residue was introduced.

As other cationic macromolecules, polyalkylenimines, particularly polyethylenimines (PEIs), are known (N. Oku et al., J. Biochemi. 100, 935-944, 1986, N. Oku et al., Biochemistry 26, 8145-8150, 1987, Suh et al., Bioorganic Chem. 22, 318-327, 1994). A polyethylenimine is a linear or branched polymer molecule comprising a protonated amino group in the molecule, through which a DNA molecule can be bound, and since no cell target substances or cell membrane denaturing agents have to be used, is applied for the introduction of genes (WO96/02655, O. Boussif et al., Proc. Natl. Acad, Sci. USA 92, 7297-7301, 1995, B. Abdallah et al., Hum. Gene Ther. 7, 1947-1954, 1996, Lambert et al., Mol. Cell Neurosci.

7, 239-246, 1996, R. Kircheis et al., Gene Ther. 4, 409-418, 1997, A. Baker et al., Gene Ther. 4, 773-782, 1997, A Baker et al., Nucleic Acids Res. 25, 1950-1956, 1997, Durmort et al., Gene Ther. 4, 808-814, 1997, Tang et al., Gene Ther. 4, 823-832, 1997, A. Boletta et al., Hum. Gene Ther. 8, 1243-1251, 1997, Ferrari et al., Gene Ther. 4, 1100-1106, 1997). Demeneix et al. have reported a hypothesisis regarding the mechanism of introducing polyethylenimine into cells (Artificial Self-Assembling Systems for Gene delivery, ed. by P. L. Felgner et al., p. 146-151, ACS conference proceeding series, 1996).

On the other hand, among cationic liposomes (or a lipoplex) constitutive lipids showing gene introduction ability, the above transfectum [J. -P. Behr et al., Proceedings of National Academy of Science of United States of America 86, 6982, 1989, or DOGS (PROMEGA)], LipofectAMINE [(Gibco BRL), or DOSPA, P. H. Nelson et al., FOCUS 15, 73 1993], CELLFECTIN [(Gibco BRL), or TM-TPS, Artificial Self-Assembling Systems for Gene Delivery ed. by P. L. Felgner et al., p. 169-176, ACS Conference Proceeding Series, 1996], GL67 (or #67), analogous compounds (E. R. Lee et al., Human Gene Therapy 7, 1701, 1995)], RPR 120535 relating compounds (WO97/18185, G. Byk et al., Tetrahedron Lett., 38, 3219, 1997), etc., are known as compounds comprising a spermine structure in the polar group.

However, a polyethylenimine to which a hydrophobic group has been introduced, or polyethylenimine-related constitutive analogues which do not comprise molecular weight distributions have not been reported as carriers for introducing genes. Gene delivery by a single derivative based on a partial structure in which spermines are linked in a polymolecular manner, has also not been reported yet.

Disclosure of the Invention

An objective of the present invention is to provide a composition for the introduction of negatively charged substances into cells, which has a high gene-transfer efficiency, no toxicity towards cells, and a cationic macromolecule as the constitutive

ingredient, and a method for introducing a negatively charged substance into cells using the composition.

To solve the above problems, the present inventors constructed a novel carrier, which comprises a polyalkylenimine as a constituent, into which multiple hydrophobic groups have been introduced, and studied the introduction of genes into cells using the carrier. They found that a gene could be introduced into cells with a high transfer efficiency and extremely low toxicity by using this carrier.

Specifically, the present inventors introduced multiple cetyl groups into a polyethylenimine to prepare the cetylated polyethylenimine shown in Figure 1, and prepared a carrier composition by mixing to the polyethylenimine, a phosphatidylethanolamine or a phosphatidylcholine, which are phospholipids. The obtained composition was mixed with a plasmid into which a fluorescent protein (GFP) was introduced, and the formed complex was introduced into COS-1 gene to examine the gene transfer efficiency by measuring fluorescent intensity and cell toxicity by measuring the cell density of living cells. As a result, the use of the carrier composition consisting of the cetylated-polyethylenimine as a constitutive ingredient markedly increased the gene transfer efficiency and lowered extremely the toxicity against cells in comparison with the use of ribosomes that have been conventionally used.

Subsequently, the present inventors synthesized 9 different kinds of compounds that differed in molecular weight of polyethylenimines, length and introduction rate of alkyl groups and examined the relationship with gene expression (Figures 7 and 8). As a result, the examination of these branched polyethylenimines revealed that a molecular weight of about 600 was suitable for gene expression *in vitro* and the examination of alkyl groups revealed that a cetyl group was appropriate (Table 1). Moreover, it was found that this compound is hardly influenced by serum in the process of gene transfer. When the cetylated polyethylenimine (the molecular weight of polyethylenimine is about 600) was fused directly with a DNA to

form a complex, and introduced into HepG2 cells in the presence of 50% serum, the gene expression was tenfold or higher in comparison with LipofectAMINE conventionally known as an effective gene transfer reagent (Table 1). When used as a liposome, the gene expression was two hundred times or higher against normal bovine brain vascular endothelial cells (BBEC) (Table 2) in comparison with LipofectAMINE (Table 2).

The structure of the above compound is heterogeneous as commercially available polyethylenimine having a molecular weight distribution is the starting material. Moreover, synthetically and strictly controlling the introduction rate of alkyl groups is difficult, and the binding site of these groups cannot be fixed. Therefore, novel compounds in which the structure can be fixed were synthesized. One group had three kinds of alkylated linear ethylenimines using tetraethylenpentamine as the staring material (Fig. 11), the other group had 5 kinds of alkylated linear spermines using spermine as the starting material (Fig. 16). The gene expression in these novel compounds was high as expected, and the compounds into which a cetyl group was introduced were superior in all cases. Against HepG2 cells in the presence of serum, cetylated linear ethylenimines and cetylated linear spermine showed ten times or higher gene expression (Tables 3 and 4). In the absence of serum, the octylated linear spermine showed a gene expression as high as the cetylated linear spermine (Table 4).

The above was the result of the gene introduction ability *in vitro*. In general, in the case of non-viral vectors, results obtained *in vitro* do not reflect those obtained *in vivo*, the gene expression *in vivo* being extremely low. For example, the gene expression in the above cationic liposome (lipofectAMINE, DC-Chol/DOPE) following intraneoplastic administration into a tumor-bearing mouse model, is reportedly lower than naked DNA (Gene Ther. 1996, 3, 542-548). Therefore, a tumor-bearing mouse model to which human pancreatic adenocarcinoma and human glioblastoma were subcutaneously transplanted was prepared, and the gene expression against this model was examined. Similarly

to the above, octylated linear ethylenimines and octylated linear spermine derivatives in addition to cetylated linear ethylenimines and cetylated linear spermines, showed obviously stronger gene expression in comparison with naked DNA (Table 5).

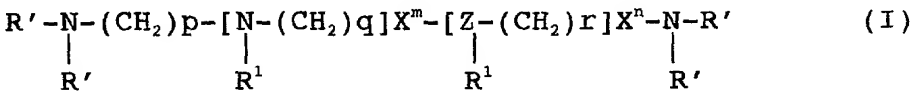
The present inventors have found that alkylated branched polyethylenimines, especially when used as liposomes, showed superior gene introduction ability in the presence of serum. Based on this, other alkylated linear ethylenimines and alkylated linear spermines were synthesized to examine the gene expression. Both cetyl and octyl-introduced derivatives showed a superior gene expression *in vivo*, when used as a complex with a DNA.

Specifically, the present invention relates to a composition comprising polyalkylenimine into which multiple hydrophobic groups have been introduced, or its salt as an ingredient, and a method for introducing genes using the composition, and more specifically:

(1) a composition comprising polyalkylenimine having two or more hydrophobic groups, or its salt,

(2) the composition of (1), wherein the hydrophobic group is a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group or a phospholipid residue,

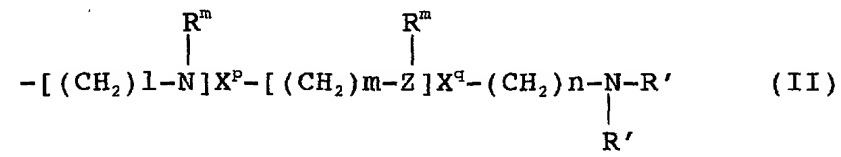
(3) the composition of (1), wherein the polyalkylenimine having two or more hydrophobic groups is a compound represented by formula (I):



(I)

wherein the base skeleton may contain an amide bond; Z represents a carbon or nitrogen atom; R′ represents hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid residue; two R′s binding to the same nitrogen atom can be identical or different; a side chain R_1 is hydrogen, a cholesterol residue, a saturated or

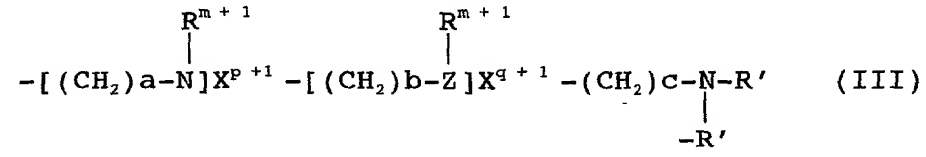
unsaturated alkyl group, a saturated or unsaturated acyl group, or a saturated or unsaturated acyloxycarbonyl group, a phospholipid residue, or below formula (II); and p, q, r, X^n, X^m represent arbitrary natural numbers:



$$-[(CH_2)l-N]X^p-[(CH_2)m-Z]X^q-(CH_2)n-N-R' \qquad (II)$$

(with R^m on the N of the $[(CH_2)l-N]$ unit, R^m on the Z of the $[(CH_2)m-Z]$ unit, and R' on the N of the $(CH_2)n-N$ unit)

wherein the base skeleton and a side chain R^m may contain an amide bond; Z represents a carbon or nitrogen atom; R' represents hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid residue; two R's binding to the same nitrogen atom can be identical or different; a side chain R^m is hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, a phospholipid residue, or below formula (III); and l, m, n, X^p, X^q represent arbitrary natural numbers:



$$-[(CH_2)a-N]X^{p+1}-[(CH_2)b-Z]X^{q+1}-(CH_2)c-N-R' \qquad (III)$$

(R^{m+1} on the N of the $[(CH_2)a-N]$ unit, R^{m+1} on the Z of the $[(CH_2)b-Z]$ unit, and $-R'$ on the N of the $(CH_2)c-N$ unit)

wherein the base skeleton and the base skeleton of the side chain R^{m+1} may contain an amide bond; Z represents a carbon or nitrogen atom; R' represents hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid residue; two R's binding to the same nitrogen atom can be identical or different; and a, b, c, X^{p+1}, X^{q+1} represent arbitrary natural numbers.

(4) The composition of any one of (1) to (3), comprising the repeating structure of formula (IV) in the base skeleton:

$$- CH_2 - CH_2 - NH - \qquad (IV).$$

(5) The composition of (4), wherein two to five molecules of tetraethylenepentamine are linked in a linear manner,

(6) The composition of (5), wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups,

(7) The composition of (5), wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl groups,

(8) The composition of (4), wherein the structure containing the formula (IV) are linked in a branched manner,

(9) The composition of (8), wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups,

(10) The composition of (8), wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl groups,

(11) The composition of any one of (1) to (3), wherein the base skeleton contains a spermine structure,

(12) The composition of (11), wherein two to five molecules of spermines are linked in a linear manner,

(13) The composition of (11), wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups,

(14) The composition of (11), wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl groups,

(15) The composition of (11), wherein the spermine structure is linked in a branched manner,

(16) The composition of (14), wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups,

(17) The composition of (14), wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl groups,

(18) The composition of any one of (1) to (17), further comprising phospholipid,

(19) The composition of (18), wherein the phospholipid is a neutral or basic phospholipid,

(20) The composition of (19), wherein the phospholipid comprises phosphatidylethanolamine, or phosphatidylcholine skeleton,

(21) The composition of (20), wherein the phospholipid is dioleylphosphotidylethanolamine, or phosphatidylcholine,

(22) A complex comprising a physiologically active substance comprising a negative charge and a composition of any one of (1) to (21),

(23) The complex of (22), in which the physiologically active substance comprising a negative charge is a nucleic acid or a derivative thereof,

(24) A method for introducing a physiologically active substance comprising a negative charge to cells, said method comprising a step of contacting the complex of (22) or (23) with cells,

(25) A kit for preparing the composition of any one of (19) to (21), comprising phospholipid and polyalkylenimine having two or

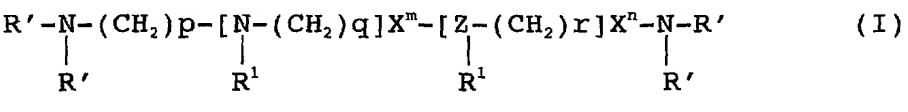
more hydrophobic groups per molecule or its salt.

The present invention relates to a composition comprising polyalkylenimine in which two or more hydrophobic groups have been introduced per molecule, or its salt. In the present invention, "polyalkylenimine" is meant by a polymer molecule comprising a protonated amino group, and may be a linear or branched. An alkylenmonomer, a constitutive unit of polyalkylenimine, is preferably a lower alkylenimine comprising 1 to 10 carbon atoms, and more preferably a lower alkylenimine with 1 to 3 carbon atoms from the viewpoint of water-solubility. From the viewpoint of convenience of synthesis, particularly, polyethylenimine or spermine is preferable. A polyethylenimine can be prepared by the methods known to one skilled in the art (for example, refer to Examined Published Japanese Patent Application (JP-B) No. Sho 49-331230, JP-B Sho 43-8828, U.S. Patent No. 4,032,480, and U.S. Patent No. 4,467,115]. Alternatively, commercial products [for example, polyethylenimine (molecular weight 600), product name EPOMINE (Nippon Shokubai), product name ExGen 500 (Euromedex), tetraethylenepentamine, etc.], may be used. The average molecular weight among polyethylenimines used in the present invention is usually 200 to 1,000,000, and preferably 300 to 500,000, and more preferably 500 to 100,000. Commercial products of spermine can also be used.

Any hydrophobic group that improves affinity between polyalkylenimine and phospholipid can be used as a hydrophobic group that is introduced into polyalkylenimine. For example, a cholesterol residue, a saturated or unsaturated alkyl group, or a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycalbonyl group, or a phospholipid residue can be used. Preferably an octyl group, a cetyl group, a stearyl group, and an oleyl group can be used. To enhance the transfer efficiency of negatively charged substance into cells, at least two hydrophobic groups are introduced into a polyalkylenimine molecule. Introduction of too many hydrophobic groups lowers the water-solubility of polyalkylenimine, therefore is not preferable. One skilled in the art can conveniently choose the

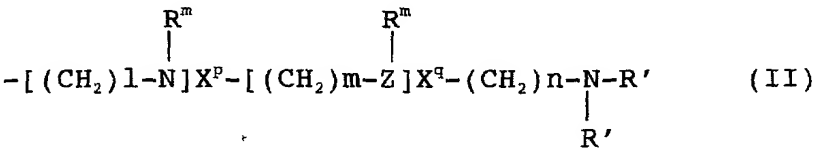
appropriate number of hydrophobic groups in a molecule. The hydrophobic group introduced into polyalkylenimine may bind to polyalkylenimine through a spacer. A neutral water-soluble molecule is preferable as a spacer. For example, an amino acid, a peptide, a polyamino acid, a protein, a sugar, a synthetic macromolecule, such as a polyethylene glycol, a polyvinyl alcohol, a polyvinyl pyrrolidone, a dextran derivative, etc., and their derivatives can be used. In the case of using a negatively charged substance as a carrier, polyalkylenimine may form a salt. As a salt of polyalkylenimine, for example, hydrochloride, sulfate, phosphate, carbonate, formate, oxalate, citrate, succinate, and such, can be used, but is not limited thereto.

The formula (I) indicates the preferable embodiment of polyalkylenimine of the present invention:



(I)

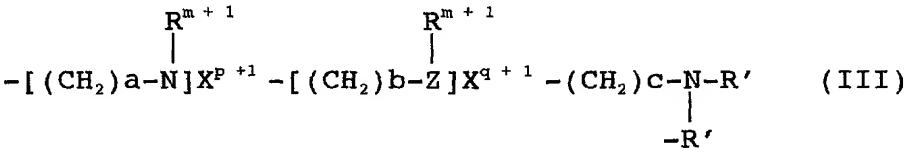
wherein the base skeleton may contain an amide bond; Z represents a carbon or nitrogen atom; R′ represents hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid group; two R's binding to the same nitrogen atom can be identical or different; a side chain R_1 is hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, or a saturated or unsaturated acyloxycarbonyl group, a phospholipid residue, or below formula (II); and p, q, r, X^n, X^m represent arbitrary natural numbers:



(II)

wherein the base skeleton and the side chain R^m may contain an amide bond; Z represents a carbon or nitrogen atom; R′ represents

hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid group; two R's binding to the same nitrogen atom can be identical or different; a side chain R^m is hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, or a saturated or unsaturated acyloxycarbonyl group, a phospholipid residue, or below formula (III); and l, m, n, X^p, X^q represent arbitrary natural numbers:



$$-[(CH_2)a-N(R^{m+1})]X^{p+1}-[(CH_2)b-Z(R^{m+1})]X^{q+1}-(CH_2)c-N(-R')-R' \qquad (III)$$

wherein the base skeleton and the side chain R^{m+1} may contain an amide bond; Z represents a carbon or nitrogen atom; R' represents hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid residue; two R's binding to the same nitrogen atom can be identical or different; and a, b, c, X^{p+1}, X^{q+1} represent arbitrary natural numbers.

The polyalkylenimine can comprise the repeating structure of the following formula in the base skeleton:

$$-CH_2-CH_2-NH- \qquad (IV)$$

and preferably 2 to 5 molecules of tetraethylenepentane are linked in a linear manner.

More preferably, any two or more of side chain R', R^l, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups, and more preferably, any two or more of side chain R', R^l, R^m, or R^{m+1} comprise

a group selected from the group consisting of butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl groups.

In the polyalkylenimine, the structure comprising the formula (IV) may be linked in a branched manner.

In the polyalkylenimine of the invention, the basic skeleton may contain a spermine structure. Preferably, 2 to 5 spermine molecules are linked in a linear chain. The spermine structure may be linked in branched manner.

The polyalkylenimine prepared in this manner into which multiple hydrophobic groups are introduced, can be used as a carrier to transport negatively charged substances by itself. Moreover, by mixing with a phospholipid to form a liposome, it can be used as a carrier. The polyalkylenimine into which the hydrophobic group was introduced presumably exists stably in the liposome as the hydrophobic group comprises a feature of being stable inside of the phospholipid. As a phospholipid used for the formation of the liposome, neutral or acidic lipid that does not interact with a physiologically active substance comprising negative charge by itself is preferable. Phospholipids may derive from nature or be synthetic. As an alkyl side chain of the phospholipid used in the present invention, one with 12 to 18 carbon atoms, or an oleyl group are preferable. As a phospholipid, for example, dioleoylphosphatidylethanolamine comprising the phosphatidylethanolamine skeleton, or phosphatidylcholine comprising the phosphatidylcholine skeleton (for example, derived from egg-yolk, soybean, or synthetic one), are suitably used. Any mixing ratio of phospholipid and polyethylenimine can be used as long as the mixture is positively charged in the range of comprising electrical affinity to a negatively charged substance. One skilled in the art can conveniently choose the preferable ratio of mixing.

The present invention also relates to a complex comprising a physiologically active substance with a negative charge and the above composition. Any physiologically active substance with

multiple negative charges can be used as the physiologically active substance with a negative charge. For example, a nucleic acid and its derivative can be used. As a nucleic acid, circular or linear, single or double stranded deoxyribonucleic acid, or ribonucleic acid can be used. As a derivative of nucleic acids, for example, phosphorothioate, phosphorodithioate, etc., can be given. In the complex of the present invention, the mixing ratio of the above composition against the physiologically active substance comprising a negative charge is preferably 1/100 equivalent to 20 equivalents at the charge ratio. One skilled in the art can conveniently choose an appropriate mixing ratio. The particle size of the complex is preferably 200 nm or less, and more preferably 100 nm or less. In the preparation of the complex of the present invention, another adjuvant is not necessary, however, as an adjuvant, amphipathic molecules, etc., such as phosphatidylethanolamine or phosphatidylcholine, fatty acid, etc., can be used.

In the case of using polyalkylenimine of the present invention in the transport of a negatively charged substance by itself, a complex is formed by directly mixing the negatively charged substance, and the negatively charged substance can be introduced into cells by following known methods (O. Boussif et al., Proc Natl. Acad. Sci. USA 92, 7297-7301, 1995). While in the case of forming a liposome by mixing polyalkylenimine of the present invention with a phospholipid, polyalkylenimine of the present invention is mixed with a phospholipid, such as dioleylphosphatidylethanolamine (DOPE, Nippon Seika), or phosphatidylcholine (eggPC, Nippon Seika) to prepare an aqueous dispersion of the liposome by known methods (N. Oku et al., Biochim. Biophys. Acta, 1280, 149-154, 1996). In this case, the liposome may contain a sterol group, such as cholesterol, as a membrane stabilizer, and tocopherol, vitamin E, etc. as an antioxidant. In the case of lipidmicrosphere, a target lipidmicrosphere can be obtained by mixing polyalkylenimine of the present invention, soybean oil, and detergent by means of known methods (F. Liu et al., Pharmaceutical Res. 13, 1642-1646, 1996). A negatively

charged substance can be introduced into cells by mixing a lipidmicrosphere with negatively charged substances, and contacting with cells.

The present invention also relates to a kit for preparing liposome, comprising phospholipid and polyalkylenimine in which two or more hydrophobic group have been introduced per molecule or its salt. The kit of the present invention can contain the above membrane stabilizer, and antioxidant as well as phospholipid and a polyalkylenimine having two or more hydrophobic per molecule. The final product of the phospholipid sample and the polyalkylenimine sample contained in the kit of the present invention can be cooled, frozen, or lyophilized. In the case of the lyophilized product, sorbitol, sucrose, amino acid and various proteins, etc., can be contained as a stabilizer.

Brief Description of the Drawings

Figure 1 shows the structure of polyethylenimine used in Examples into which hydrophobic groups had been introduced.

Figure 2 shows GFP gene expression when the charge ratio of a plasmid and a liposome was varied in the case of using the composition of the present invention, and a conventional liposome. In the figure, PCL indicates tricetyl-PEI: DOPE (0.65:1, molar ratio; molecular weight of polyethylenimine, 600). Similarly, DMRIE:DOPE (1:1, molar ratio), and DOTAP: DOPE (1:1, molar ratio) are shown, and COS-1 cell was used. The error-bar over the plot indicates ±SD.

Figure 3 shows the effect of phospholipid on gene expression. In the figure, DOPE-PCL indicates tricetyl-PEI:DOPE (0.65:1, molar ratio), eggPC-PCL indicates tricetyl-PEI: eggPC (0.65:1, molar ratio).

Figure 4 shows cytotoxicity when the concentration of the liposome of the present invention alone or of the plasmid complex was changed. In the figure, PCL indicates tricetyl-PEI:DOPE (0.65:1, molar ratio; molecular weight of polyethylenimine, 600). COS-1 cell was used. The error bar indicates the plus value of ±SD.

Figure 5 shows the cytotoxicity when the concentration of the conventional liposome (DMRIE) alone or of the plasmid complex was changed. In the figure, DMRIS Lipo. indicates DMRIE: DOPE (1:1, molar ratio). COS-1 cell was used. The error-bar indicates the plus value of ±SD.

Figure 6 shows the cytotoxicity when the concentration of the conventional liposome (DOTAP) alone or of the plasmid complex was changed. In the figure, DOTAP Lipo. indicates DOTAP:DOPE (1:1, molar ratio). COS-1 cell was used. The error-bar indicates the plus value of ±SD.

Figure 7 shows the structure of an alkylated branched polyethylenimine using polyethylenimine with a molecular weight of 600 as a starting material.

Figure 8 shows the structure of an alkylated branched polyethylenimine using polyethylenimine with a molecular weight of 1,800 as a starting material.

Figure 9 shows the synthesis route (1) of an alkylated linear ethylenimine.

Figure 10 shows the synthesis route (2) of an alkylated linear ethyleneimine.

Figure 11 shows the structure of alkylated linear ethylenimines.

Figure 12 shows the synthesis route (1) of an alkylated linear spermine.

Figure 13 shows the synthesis route (2) of an alkylated linear spermine.

Figure 14 shows the synthesis route (3) of an alkylated linear spermine.

Figure 15 shows the synthesis route (4) of an alkylated linear spermine.

Figure 16 shows the structure of the alkylated linear spermines.

Best Mode for Carrying out the Invention

The present invention is illustrated in detail by Examples, but is not to be construed as being restricted thereto.

Example 1: Purification of polyethylenimine

Commercially available polyethylenimine (molecular weight 600, product name: EPOMINE SP-006, Nippon Shokubai) (2 g) was dissolved in 100 ml of distilled water and ultrafiltered through a ultrafiltration membrane of a molecular cut off of 500 (UH-05, Toyo Paper Filter, the ultrafiltration device was manufactured by Amicon, stirring type cell model 8,400), under 2 to 3 kg/cm^2 nitrogen stream, with 1000 ml of distilled water. The ultrafiltrate in which impurities were removed (about 20 to 30 ml) was lyophilized to obtain purified polyethylenimine.

Example 2: Synthesis of cetylated polyethylenimine

In 20 ml of chloroform, 1 g of purified polyethylenimine (molecular weight: 600) and 1.62 g of cetyl bromide were mixed (molar ratio, 1:2), 1 ml of triethylamine was added thereto and refluxed. Subsequently, unreacted polyethylenimine was removed using an ultrafiltraion membrane with a molecular cut off of 1000 (YM1, Amicon). Yield of lyophilized cetylated polyethylenimine was 70.4%. The number of the introduced cetyl groups on cetylated polyethylenimine was confirmed by NMR. Specifically, the proton ratio of cetyl group at around 1.3 ppm and polyethylenimine at around 2.5 to 3.5 ppm supported that 3 molecules of cetyl groups bound to one molecule of polyethylenimine. Figure 1 shows the synthesized polyethylenimine.

Example 3: Preparation of liposome

To prepare a liposome, a chloroform solution of 10 mM cetylated polyethylenimine and a chloroform solution of 10 mM dioleylphosphatidylethanolamine (DOPE, Nippon Seika) were prepared beforehand. The lipid composition used was cetylated polyethylenimine/DOPE = 0.65/1 (molar ratio). This lipid was dissolved in chloroform in a pear-shaped flask, chloroform was removed under reduced pressure by a rotary evaporator, and a lipid film was prepared. This lipid film was dried under high vacuum for one hour, hydrated with DMEM (Gibco BRL) and adjusted to 1

mM. Freeze-thawing was repeated three times and the liposome was treated by ultrasonication for 10 min in a bath-type ultrasonicator. Even though liposomes are stable, they were however, prepared when necessary.

As a comparative example, 1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide (DMRIE), or 1,2-dioleyloxy-3-(trimethylammonio)propane (DOTAP) was used in stead of cetylated polyethylenimine to obtain liposomes in the same manner as in the above.

Example 4: Preparation of plasmid DNA/liposome complex

pEGFP-C1 (Clontech) was used as the plasmid DNA. This plasmid encodes GFP (green fluorescent protein) as a reporter gene to quantify the gene expression by measuring fluorescent intensity. The plasmid was prepared by *E. coli*, purified by the cesium chloride density-gradient centrifugation method, dissolved in TE buffer (pH 7.5) to 2 μg/ml. The quantity of plasmid DNA was kept constant, transferred to a 1.5-ml Eppendorf tube and 1 mM liposome solutions with various ratios were added thereto. DMEM was added to adjust the volume to 100 μl and incubated at room temperature for 20 min.

Example 5: Introduction of genes

COS-1 cells were spread in a 35-mm dish to 1 x 10^5 cells per dish, and cultured at 37°C in the presence of 5% CO_2 overnight. The cells were washed with DMEM twice. The prepared liposome/plasmid DNA complex was diluted with serum-free DMEM and added to cells to adjust the total volume to 250 μl. The cells were incubated at 37°C in the presence of 5% CO_2 for three hours. After 3 hours, the liposome/plasmid DNA complex solution was removed, washed with serum-free DMEM twice, 2 ml of 10% FBS-DMEM was added thereto, and the cells were incubated for 48 hours.

Example 6: Quantification of the expression of the introduced genes (GFP)

The medium was removed from the above cells incubated for

48 hours. The cells were washed with serum-free DMEM twice. Triton X 100 solution was added thereto to a final concentration of 1%, and the cells were incubated at room temperature for 30 min for solubilization. The cell suspension was transferred to an Eppendorf tube and centrifuged at 3,000 rpm for 10 min. The amount of gene expression was determined by measuring fluorescent intensity at Ex 493 nm, and Em 510 nm. Figure 2 shows the result. When the composition of the present invention was used, the expression was twice or higher in comparison with the controls of DMRIE and DOTAP.

Example 7: Gene expression in the case of using the comparative liposome

The expression of the introduced gene was measureed in the same manner as in Examples 1 to 6, except for using egg-yolk-derived phosphatidylcholine (eggPC) instead of DOPE in Example 3. Figure 3 shows the result. In the case of using eggPC, the gene expression was similar to that in DOPE.

Example 8: Examination of cytotoxicity

One milliliter of COS-1 cells were added to each well of a 24-well plate (Corning), and incubated at 37°C in the presence of 5% CO_2 overnight until confluency. Each well was washed with DMEM twice, and 200 μl of the prepared plasmid DNA/liposome complex solution of various concentrations was added into each well, incubated at 37°C in the presence of 5% CO_2 for three hours. In the same manner, DMEM-only and liposome solution-only were examined as controls.

Example 9: Measurement of cytotoxicity

Cytotoxicity was measured by using AlamerBlue (Biosource International, imported by Iwaki Glass). Each sample solution was removed and 200 μl of serum-free DMEM and 50 μl of AlamerBlue diluted into 1/5 were added to each well. After 1 hour of incubation at 37°C in the presence of 5% CO_2, the solution in each well was transferred to a 1.5-ml Eppendorf tube, and 750 μl of

PBS(-) was added thereto to make a total volume of 1 ml. Fluorescent intensity was measured at Ex 535 nm and Em 583 nm. Figures 4, 5, and 6 show the results. In the case of using the composition of the present invention, the cytotoxicity was extremely lowered, especially at the high concentration of the complex solution, compared with using the conventional liposome.

Example 10: Synthesis of alkylated branched polyethylenimine derivative

To examine the effect of chain length of an alkyl group on gene transfer ability, the alkyl groups were introduced into polyethylenimine of a molecular weight of 600 [hereafter referred to as PEI (6), Compound 1, compound name: p6] using the same procedures in Examples 1 and 2 (Figures 7 and 8).

Synthesis of Compound 2

Synthesis of Compound name, P6D20.3 (Degree of alkylation: 20.3%)

Polyethylenimine and PEI (6) (5.0 g) were dissolved in 20 ml of ethanol, and 3.69 g (3.45 ml) of decyl bromide was slowly added thereto. The reaction was effected at 75°C while the solution was being stirred with a nitrogen flow. After 8 hours, quenching of decyl bromide was confirmed by TLC to complete the synthesis. Subsequently 150 ml of distilled water was added thereto and ultrafiltration (Amicon YM1) was conducted for purification. Polymers were obtained by removing water by freeze-drying. Introduction of 20.3% decyl groups was identified from a 42.2% yield; ^{1}H NMR ($CDCL_3$) δ : 0.85 (9H, t, CH_3-), 1.30 (36H, m, CH_2 of decyl), 2.70 (54H, br, $-C_2H_4-N-$), 3.20 (14H, m, -NH-), and 2.70 proton ratio.

Synthesis of compound 3

Synthesis of compound name, P6L18.4 (degree of alkylation: 18.4%)

PEI (6) (2.0 g) was dissolved in 10 ml of ethanol, and 1.66 g (1.60 ml) of lauryl bromide was slowly added thereto. The reaction was effected at 75°C while the solution was being stirred with a nitrogen flow. After 8 hours, quenching of decyl bromide

was confirmed by TLC to complete the synthesis. Subsequently 150 ml of distilled water was added thereto and ultrafiltration (Amicon YM1) was conducted for purification. Polymers were obtained by removing water by freeze-drying. Introduction of 18.4% lauryl groups was identified from a 68.4% yield; ^{1}H NMR (D_2O) δ: 0.80 (8H, t, CH_3-), 1.20 (46H, m, CH_2 of lauryl), 2.60 (54H, br, -C_2H_4-N-), and 2.60 proton ratio.

Synthesis of compound 4

Synthesis of compound name, P6M18.8 (degree of alkylation: 18.8%)

PEI (6) (5.0 g) was dissolved in 20 ml of ethanol, and 4.63 g (4.97 ml) of myristyl bromide was slowly added thereto. The reaction was effected at 75°C while the solution was being stirred with a nitrogen flow. After 8 hours, quenching of myristyl bromide was confirmed by TLC to complete the synthesis. Subsequently 150 ml of distilled water was added thereto and ultrafiltration (Amicon YM1) was conducted for purification. Polymers were obtained by removing water by freeze-drying. Introduction of 18.8% myristyl groups was identified from a 54.8% yield; ^{1}H NMR ($CDCl_3$) δ: 0.90 (8H, t, CH_3-), 1.30 (55H, m, CH_2 of myristyl), 2.70 (55H, br, -C_2H_4-N-), 3.55 (20H, m, -NH-), and 2.70 proton ratio.

Synthesis of compound 5

Synthesis of compound name, P6C17.5 (degree of alkylation: 17.5%)

PEI (6) (1.28 g) was dissolved in 10 ml of ethanol, and 1.30 g (1.30 ml) of cetyl bromide was slowly added thereto. The reaction was effected at 75°C while the solution was being stirred with a nitrogen flow. After 8 hours, quenching of cetyl bromide was confirmed by TLC to complete the synthesis. Subsequently 150 ml of distilled water was added thereto and ultrafiltration (Amicon YM1) was conducted for purification. Polymers were obtained by removing water by freeze-drying. Introduction of 17.5% cetyl groups was identified from a 82.6% yield; ^{1}H NMR ($CDCl_3$) δ: 0.88 (8H, t, CH_3-), 1.25 (60H, m, CH_2 of cetyl), 2.70 (44H, br, -C_2H_4-N-), 3.90 (8H, m, -NH-) δ: 0.88, and 2.70 proton ratio.

Synthesis of compound 6

Synthesis of compound name, P6C24.5 (degree of alkylation: 24.5%)

PEI (6) (1.0 g) was dissolved in 10 ml of ethanol, and 1.02 g (1.02 ml) of cetyl bromide was slowly added thereto. The reaction was effected at 75°C while the solution was being stirred with a nitrogen flow. After 8 hours, quenching of cetyl bromide was confirmed by TLC to complete the synthesis. Subsequently 150 ml of distilled water was added thereto and ultrafiltration (Amicon YM1) was conducted for purification. Polymers were obtained by removing water by freeze-drying. Introduction of 24.5% cetyl groups was identified from a 85.5% yield; ^{1}H NMR ($CDCl_3$) δ: 0.88 (10H, t, CH_3-), 1.25 (75H, m, CH_2 of cetyl), 2.65 (45H, br, -C_2H_4-N-), 4.88 (22H, m, -NH-), and 2.65 proton ration.

Synthesis of compound 7

Synthesis of compound name, P6S21.1 (degree of alkylation: 21.1%)

PEI (6) (5.0 g) was dissolved in 20 ml of ethanol, and 5.57 g (5.71 ml) of stearyl bromide was slowly added thereto. The reaction was effected at 75°C while the solution was being stirred with a nitrogen flow. After 8 hours, quenching of cetyl bromide was confirmed by TLC to complete the synthesis. Subsequently 150 ml of distilled water was added thereto and ultrafiltration (Amicon YM1) was conducted for purification. Polymers were obtained by removing water by freeze-drying. Introduction of 21.1% stearyl groups was identified from a 86.0% yield; ^{1}H NMR ($CDCl_3$) δ: 0.90 (9H, t, CH_3-), 1.30 (75H, m, CH_2 of stearyl), 2.70 (57H, br, -C_2H_4-N-), 3.70 (17H, m, -NH-), and 2.70 proton ratio.

Synthesis of compound 8

Synthesis of compound name, P6S11.3 (degree of alkylation: 11.3%)

PEI (6) (5.0 g) was dissolved in 20 ml of ethanol, 2.78 g (2.85 ml) of stearyl bromide was slowly added thereto. The reaction was effected at 75°C while the solution was being stirred with a nitrogen flow. After 8 hours, quenching of cetyl bromide was confirmed by TLC to complete the synthesis. Subsequently 150

ml of distilled water was added thereto and ultrafiltration (Amicon YM1) was conducted for purification. Polymers were obtained by removing water by freeze-drying. Introduction of 11.3% stearyl groups was identified from a 53.3% yield; ^{1}H NMR ($CDCl_3$) δ: 0.90 (5H, t, CH_3-), 1.25 (35H, m, CH_2 of stearyl), 2.60 (56H, br, $-C_2H_4-N-$), and 2.60 proton ratio.

In the same manner, a cetyl group (C=16) was introduced into polyethylenimine with a molecular weight of 1,800 (product name: EPOMINE SP-018) [refer to as PEI (18)] to synthesize two kinds of compounds with different introduction rates.

Synthesis of compound 9

Synthesis of compound name, P18C12.3 (degree of alkylation: 12.3%)

PEI (6) (1.50 g) was dissolved in 10 ml of ethanol, and 0.87 g (0.87 ml) of cetyl bromide was slowly added thereto. The reaction was effected at 75°C while the solution was being stirred with a nitrogen flow. After 8 hours, quenching of cetyl bromide was confirmed by TLC to complete the synthesis. Subsequently 150 ml of distilled water was added thereto and ultrafiltration (Amicon YM1) was conducted for purification. Polymers were obtained by removing water by freeze-drying. Introduction of 12.3% cetyl groups was identified from a 84.0% yield; ^{1}H NMR ($CDCl_3$) δ: 0.88 (15H, t, CH_3-), 1.25 (117H, m, CH_2 of cetyl), 2.70 (170H, br, $-C_2H_4-N-$), 3.70 (23H, m, -NH-), and 2.70 proton ratio.

Synthesis of compound 10

Synthesis of compound name, P18C20.6 (degree of alkylation: 20.6%)

PEI (6) (1.0 g) was dissolved in 10 ml of ethanol, and 1.19 g (1.19 ml) of cetyl bromide was slowly added thereto. The reaction was effected at 75°C while the solution was being stirred with a nitrogen flow. After 8 hours, quenching of cetyl bromide was confirmed by TLC to complete the synthesis. Subsequently 150 ml of distilled water was added thereto and ultrafiltration (Amicon YM1) was conducted for purification. Polymers were

obtained by removing water by freeze-drying. Introduction of 20.6% cetyl groups was identified from a 92.8% yield; ^{1}H NMR ($CDCl_3$) δ: 0.88 (26H, t, CH_3-), 1.25 (207H, m, CH_2 of cetyl), 2.70 (155H, br, $-C_2H_4-N-$), 5.15 (44H, m, -NH-), and 2.70 proton ratio.

Example 11: Preparation of stock solution

Each of 9 kinds of derivatives prepared in Example 10 was dissolved in sterilized water and pH was adjusted to around 7. The compounds difficult to dissolve in water were heated at 50 °C for 15 to 30 min, pH was adjusted, and the compounds were treated by a probe-type ultrasonicator (BRONSON 20 Dutycycle, 5 min). All carrier solutions were finally filtered through a filter (DISMIC-25cs, Cellulose acetate, 0.2 μm, ADVANTEC TOYO).

The concentration of the carrier solution was expressed as molar concentration of aminenitrogen. Specifically, in the concentration of polyethylenimine with a molecular weight of 600, molecular weight of a monomer was 43, and the mol number of the positive charge at 100 mM (100 nmpl/μl) was 43 mg/ml. While the concentration of the alkylated polyethylenimine solution was calculated by correcting the molecular weight and the introduced-number of alkyl groups. The stock solution of these alkylated polyethylenimines was stored at 4°C until used.

Example 12: Method for preparing plasmid solution

β -galactosidase (LacZ) gene [derived from pCMVbeta (Clontech)] was introduced into plasmid pCAGGS (Hum Gene Ther. 1998, 9, 1701-7) to construct plasmid pCAG-LZ15. The stock solution of this plasmid (5 mg DNA/ml TE) was diluted with D-MEM (GIBCO BLR) for cell culture medium to prepare 58 μg/ml DNA solution. The mol number of the negative charge in 1 μg of DNA is 3 nmol, and the mol number of the negative charge in 58 μg of DNA is 58 μg x 3n mol = 174 nmol.

Example 13: Method for preparing each derivative carrier

The stock solution in Example 11 was prepared with D-MEM so that N/P (a ratio of N: the mol number of the positive charge

in nitrogen of the carrier/P: the mol number of the negative charge in phosphate of DNA) was 18 equivalents.

Example 14: Method for forming a DNA complex

The DNA solution in Example 12 (100 μl) was transferred to a 1.5-ml of Eppendorf tube, and 100 μl of the carrier solution in Example 13 was added thereto, and gently vortexed. The mixture was kept at room temperature for 30 min or longer to form a DNA complex (N/P = 18 equivalents). This manipulation was conducted just before gene introduction (transfection of DNA).

Example 15: *In vitro* introduction of gene into human liver carcinoma

Human liver carcinoma Hep G2 was seeded on a 24-well dish and adjusted to 30,000 cells/well 18 hours before gene introduction of a DNA. The cells were washed twice with MEM just before gene introduction. The DNA and the complex solution of Example 6 were diluted with MEM and MEM with serum (50% FBS) and adjusted to 290 ng DNA/200 μl/well, and 200 μl of this gene introduction solution was added to each well. The cells were cultured in the 5% carbonate gas incubator at 37°C for 3 hours, the gene introduction solution was removed and the cells were washed twice with MEM containing serum (10% FBS). The same medium (1 ml) was added to each well, and cultured at 37°C for 48 hours in the 5% carbonate gas incubator. In the control lipofectAMINE (GIBCO BRL, 2 mg/ml), the DNA complex was formed at the ratio of DNA : lipofectAMINE = 290 μg : 1 μg in OPTI-MEM medium, and the same amount of DNA was used.

Example 16: Measurement of Lac Z activity

The cell-culture medium in Example 14 was removed, and washed with D-PBS(-) (Nikken) twice. Cultured cell lysis agent LCβ (Product name: Pikkagene, Wako Pure Chemicals) (100 μl) was added to each well, kept at room temperature for 15 min, and treated with a compact probe-type ultrasonicator (UR-20P, Tommy, Power 10) for 5 sec to disrupt the cells. This cell lysate was transferred

from each well to a 0.5- ml Eppendorf tube, and centrifuged at 1,200 rpm for 3 min at room temperature. The supernatant was subjected to the measurement of LacZ activity.

The Lac Z activity was measured following the protocol of TROPIX, Inc. Each gene introduction experiment was triplicated and Lac Z activity was expressed by the mean light unit ±unit SD per cell protein (mg)/measurement duration (1 sec). The cell measurement of protein was by BCA method (Pierce).

Example 17: *In vitro* gene expression of an alkylated branched polyethylenimine derivative

Table 1 shows the comparison of expression of LacZ gene against HepG2 cells into which various alkylated branched polyethylenimine-DNA complexes were introduced. Each numerical value indicates a mean ± SD (n=3). The gene expression was enhanced by introducing an alkyl group into the polyethylenimine with molecular weight of 600. Gene expression in P6C24.5 and P6C17.5 into which the cetyl group (C=16) was introduced and the DNA complex of the DNA was the highest. When introduced in the absence of serum, the gene expression was 4 times higher than lipofectAMINE and when transfected in the presence of 50% serum, it was 10 times higher than LipofectAMINE.

The molecular weight of 600 showed a higher value than the molecular weight of 1800 when molecular weights of the polyethylenimines for each cetyl derivative were compared.

Table 1

Compound	Compound No.	Gene Expression LacZ (RLU/μg · sec)	
		No serum	50% Serum
P6	1	998±277	675±405
P9D20.3	2	87,740±15,127	485±216
P6L18.4	3	343,267±3,449	25,178±5,649
P6L18.8	4	193,790±24,371	307±135
P6C17.5	5	453,539±7,415	446,556±50,168
P6C24.5	6	458,970±24,796	467,869±8,685
P6S21.1	7	282,858±29,015	168,580±18,461
P6S11.3	8	196,244±17,181	119,383±19,034
P18C12.3	9	217,607±7,083	80,766±7,426
P18C20.6	10	34,336±6,861	28,585±4,520
LipofectAMINE		113,637±48,504	34,847±5,483

Example 18: Comparison of gene expression with the liposome

Gene expression of the complex of P6C24.5 and DNA was compared with that of the complex of the liposome and DNA (PCL). The composition of the liposome was P6C24.5/DOPE (1:1, molar ratio), and the method for preparation of Example 3 followed. The DNA complexes were prepared following the method of Examples 12 to 14. Normal bovine brain vessel endothelial cells (BBEC, CELLSYSTEMS, by Dainippon Pharmaceuticals), was used and the gene was introduced in the same manner as in Example 15.

Table 2 shows the result. In the table, PCL indicates P6C24.5/DOPE (1:1, molar ratio) and each numerical value indicate a mean ± SD (n=3). The gene expression in the complex of PCL and DNA was about same as P6C24.5 alone and about 10 times higher than LipofectAMINE when introduced in the absence of serum. On the other hand, when gene introduction was conducted in the presence of serum, the gene expression of the complex of PCL and DNA was 10 timer higher than P6C24.5 alone, and about 200 times higher than LipofectAMINE.

Table 2

	Charge ratio	Gene Expression LacZ (RLU/μg·sec)	
		No serum	50% serum
P6C24.5	18 eq	214,053±75,524	5,634±1,381
PCL	9 eq	192,369±24,775	78,051±5,891
LipofectAMINE		20,628±11,680	41±3

Example 19: Synthesis of alkylated linear ethylenimines

By following the synthesis route shown in Figures 9 and 10, 3 kinds of alkylated linear ethylenimines shown in Figure 11 were synthesized. The following is the synthesis method.

Synthesis of compound 12 (Figure 9)

Sodium hydride (60% mineral oil suspension, 0.462 g, 11.6 mmol) was suspended in dimethylformamide (160 ml) and dimethylformamide solution of tetraethylenepentamin-pentatosylate (Compound 11, 10.1 g, 10.5 mmol) (40 ml) was dropped thereto while cooling with ice. The solution was stirred for 1 hour at room temperature, 3-[(tert-butyldimethylsilyl)oxy]-1-bromopropane (2.92 g, 11.5 mmol) was dropped while cooling with ice. The reaction mixture was stirred at room temperature for 20 hours, and poured into water and extracted by ethyl acetate twice. The extracts were combined and washed with saturated brine, dried and concentrated. The residue was subjected to column chromatography with silica gel (500 g), and the fraction eluted with hexane-ethyl acetate (2:1) was collected. Compound 2 (3.57 g) was obtained as a white powder. Yield 30%; ^{1}H NMR ($CDCl_3$), δ: 0.024 (6H, s), 0.88 (9H, s), 1.65-1.78 (2H, m), 2.39 (3H, s), 2.41 (3H, s), 2.42 (3H, s), 2.44 (3H, s), 2.45 (3H, s), 3.10-3.45 (18H, m), 3.58 (2H, t, J=6.0 Hz), 5.39 (1H, t, J=5.9 Hz), 7.23-7.38 (10H, m), 7.65-7.83 (10H, m). FAB MS m/z: 1132 $[M+H]^+$.

Synthesis of Compound 13 (Figure 9)

Sodium hydride (60% mineral oil suspension, 0.0644 g, 1.16 mmol) was suspended in dimethylformamide (24 ml) and 6 ml of

dimethylformamide of Compound 12 (1.52 g, 1.34 mmol) was dropped thereto while cooling with ice. The solution was stirred for 1 hour at room temperature, 1-bromohexadecane (491 μl, 1.61 mmol) was dropped while cooling with ice. The reaction mixture was stirred at room temperature for 6 hours, poured into water and extracted by ethyl acetate twice. The extracts were combined and washed with saturated brine, dried and concentrated. The residue was subjected to column chromatography with silica gel (35 g), and the fraction eluted with hexane-ethyl acetate (3:1) was collected. Compound 13 (1.64 g) was obtained as a white powder. Yield 30%; ^{1}H NMR ($CDCl_3$), δ: 0.023 (6H, s), 0.80-0.95 (3H, m), 0.88 (9H, s), 1.10-1.35 (26H, m), 1.42-1.55 (2H, m), 1.64-1.80 (2H, m), 2.40 (6H, s), 2.42 (6H, s), 2.43 (3H, s), 3.01-3.11 (2H, m), 3.16-3.23 (2H, m), 3.25-3.45 (16H, m), 3.58 (2H, t, J=5.9 Hz), 7.25-7.36 (10H, m), 7.71-7.86 (10H, m). FAB MS m/z: 1356 $[M+H]^+$.

Synthesis of Compound 14 (Figure 9)

Compound 14 (1.48 g) was obtained as a white powder from Compound 12 (1.47 g, 1.30 mmol) and 1-bromododecane (373 μl, 1.56 mmol), in the same manner as in the synthesis of Compound 13. Yield 88%; ^{1}H NMR ($CDCl_3$), δ: 0.023 (6H, s), 0.80-0.95 (3H, m), 0.88 (9H, s), 1.10-1.35 (18H, m), 1.41-1.58 (2H, m), 1.64-1.80 (2H, m), 2.40 (6H, s), 2.41 (6H, s), 2.43 (3H, s), 3.01-3.11 (2H, m), 3.16-3.23 (2H, m), 3.25-3.45 (16H, m), 3.58 (2H, t, J=5.9 Hz), 7.25-7.36 (10H, m), 7.71-7.86 (10H, m). FAB MS m/z: 1300 $[M+H]^+$.

Synthesis of Compound 15 (Figure 9)

Compound 15 (0.730 g) was obtained as a white powder from Compound 12 (0.803 g, 0.709 mmol) and 1-bromooctane (147 μl, 0.851 mmol), in the same manner as in the synthesis of Compound 13. Yield 83%; ^{1}H NMR ($CDCl_3$), δ: 0.023 (6H, s), 0.87 (3H, t, J=6.6 Hz), 0.88 (9H, s), 1.10-1.35 (10H, m), 1.45-1.57 (2H, m), 1.66-1.77 (2H, m), 2.40 (6H, s), 2.42 (6H, s), 2.43 (3H, s), 3.03-3.12 (2H, m), 3.15-3.24 (2H, m), 3.23-3.45 (16H, m), 3.58 (2H, t, J=5.9 Hz), 7.26-7.35 (10H, m), 7.70-7.81 (10H, m). FAB MS m/z: 1244 $[M+H]^+$.

Synthesis of Compound 16 (Figure 9)

Tetrahydrofuran solution (1 M) of tetrabutylammonium fluoride (1.44 ml, 1.44 mmol) was dropped to tetrahydrofran solution (33 ml) of Compound 13 (1.63 g, 1.20 mmol) while cooling with ice. The reaction mixture was stirred at room temperature for 1.5 hours, diluted with water, and extracted with ethyl acetate twice. The extract was washed with water, dried and concentrated. The obtained residue was subjected to column chromatography with silica gel (30 g), and the fraction eluted with hexane-ethyl acetate (1:1) was collected. Compound 13 was obtained as a white powder (1.30 g). Yield 87%; ^{1}H NMR ($CDCl_3$), δ: 0.88 (3H, t, J=7.0 Hz), 1.15-1.34 (26H, m), 1.45-1.55 (2H, m), 1.74-1.84 (2H, m), 2.26 (1H, t, J=6.2 Hz, -OH), 2.405 (3H, s), 2.415 (3H, s), 2.422 (6H, s), 2.43 (3H, s), 3.02-3.10 (2H, m), 3.22 (2H, t, J=6.6 Hz), 3.25-3.46 (16H, m), 3.66-3.76 (2H, m), 7.26-7.35 (10H, m), 7.70-7.80 (10H, m). FAB MS m/z: 1242 $[M+H]^+$.

Synthesis of Compound 17 (Figure 9)

Compound 17 (1.21 g) was obtained as a white powder from Compound 14 (1.47 g, 1.13 mmol) and tetrahydrofuran solution (1 M) of tetrabutylammonium fluoride (1.36 ml, 1.36 mmol) in the same manner as in the synthesis of Compound 16. Yield 91%; ^{1}H NMR ($CDCl_3$), δ: 0.88 (3H, t, J=7.0 Hz), 1.16-1.35 (18H, m), 1.44-1.56 (2H, m), 1.74-1.85 (2H, m), 2.27 (1H, t, J=6.0 Hz, -OH), 2.405 (3H, s), 2.415 (3H, s), 2.423 (6H, s), 2.43 (3H, s), 3.02-3.12 (2H, m), 3.22 (2H, t, J=6.8 Hz), 3.25-3.45 (16H, m), 3.66-3.74 (2H, m), 7.26-7.35 (10H, m), 7.70-7.80 (10H, m). FAB MS m/z: 1186 $[M+H]^+$.

Synthesis of Compound 18 (Figure 9)

Compound 18 (0.513 g) was obtained as a white powder from Compound 15 (0.590 g, 0.474 mmol) and tetrahydrofuran solution (1 M) of tetrabutylammonium fluoride (0.57 ml, 0.57 mmol) in the same manner as in the synthesis of Compound 16. Yield 96%; ^{1}H

NMR ($CDCl_3$), δ: 0.87 (3H, t, J=7.0 Hz), 1.15-1.35 (10H, m), 1.44-1.56 (2H, m), 1.75-1.85 (2H, m), 2.26 (1H, t, J=6.2 Hz, -OH), 2.41 (3H, s), 2.415 (3H, s), 2.423 (6H, s), 2.43 (3H, s), 3.02-3.12 (2H, m), 3.22 (2H, t, J=6.6 Hz), 3.26-3.44 (16H, m), 3.66-3.75 (2H, m), 7.27-7.35 (10H, m), 7.70-7.80 (10H, m). FAB MS m/z: 1130 $[M+H]^+$.

Synthesis of Compound 19 (Figure 9)

Triphenylphosphine (0.988 g, 3.77 mmol) was added to methylene chloride solution (26 ml) of Compound 16 (1.30 g, 1.05 mmol) and carbon tetrabromide (1.25 g, 3.77 mmol) while cooling with ice. The reaction mixture was stirred for 20 min while cooling with ice, further stirred at room temperature for 1 hour, and dried under reduced pressure. The obtained residue was subjected to column chromatography with silica gel (60 g), and the fraction eluted with hexane-ethyl acetate (3:1) was collected. Compound 19 was obtained as a white powder (1.34 g). Yield 98%; ^1H NMR ($CDCl_3$), δ: 0.88 (3H, t, J=7.0 Hz), 1.15-1.35 (26H, m), 1.43-1.55 (2H, m), 2.07-2.18 (2H, m), 2.405 (3H, s), 2.413 (3H, s), 2.42 (6H, s), 2.43 (3H, s), 3.02-3.12 (2H, m), 3.18-3.46 (20H, m), 7.26-7.36 (10H, m), 7.70-7.82 (10H, m). FAB MS m/z: 1304 and 1306 $[M+H]^+$.

Synthesis of Compound 20 (Figure 9)

Compound 20 (1.21 g) was obtained as a white powder from Compound 17 (1.19 g, 1.10 mmol) and carbon tetrabromide (1.20 g, 3.61 mmol), triphenylphosphine (0.947 g 3.61 mmol) in the same manner as in the synthesis of Compound 19. Yield 97%; ^1H NMR ($CDCl_3$), δ: 0.88 (3H, t, J=7.0 Hz), 1.15-1.35 (18H, m), 1.44-1.56 (2H, m), 2.07-2.18 (2H, m), 2.405 (3H, s), 2.414 (3H, s), 2.42 (6H, s), 2.43 (3H, s), 3.03-3.10 (2H, m), 3.14-3.45 (20H, m), 7.26-7.36 (10H, m), 7.70-7.82 (10H, m). FAB MS m/z: 1248 and 1250 $[M+H]^+$.

Synthesis of Compound 21 (Figure 9)

Compound 21 (1.05 g) was obtained as a white powder from

Compound 18 (1.00 g, 0.886 mmol) and carbon tetrabromide (1.06 g, 3.19 mmol), triphenylphosphine (0.837 g, 3.19 mmol) in the same manner as in the synthesis of Compound 19. 99% yield; ^{1}H NMR ($CDCl_3$), δ: 0.87 (3H, t, J=7.0 Hz), 1.16-1.34 (10H, m), 1.44-1.55 (2H, m), 2.07-2.18 (2H, m), 2.405 (3H, s), 2.413 (3H, s), 2.42 (6H, s), 2.43 (3H, s), 3.03-3.10 (2H, m), 3.19-3.44 (20H, m), 7.26-7.36 (10H, m), 7.70-7.82 (10H, m). FAB MS m/z: 1192 and 1194 $[M+H]^+$.

Synthesis of Compound 22 (Figure 10)

Sodium hydride (60% mineral oil suspension, 0.0404g, 1.01 mmol) was suspended in dimethylformamide (28 ml) and dimethylformamide solution (2 ml) of tetraethylenepentamin-pentatosylate (Compound 11, 0.404 g, 0.421 mmol) was dropped thereto while cooling with ice. The solution was stirred for 1 hour at room temperature, dimethylformamide solution (4 ml) of Compound 19 (1.32 g, 1.01 mmol) was dropped while cooling with ice. The reaction mixture was stirred at room temperature for 18 hours, and poured into water and extracted with ethyl acetate twice. The extracts were combined and washed with saturated brine, dried and concentrated. The residue was subjected to column chromatography with silica gel (60 g), and the crude product eluted with hexane-ethyl acetate (2:1) was purified by the medium-pressure liquid chromatography (Rover B column). The fraction eluted with hexane-ethyl acetate (3:2) was collected. Compound 22 (1.07 g) was obtained as a white powder. Yield 75%; ^{1}H NMR ($CDCl_3$), δ: 0.88 (6H, t, J=7.0 Hz), 1.15-1.35 (52H, m), 1.43-1.56 (4H, m), 1.90-2.04 (4H, m), 2.36 (12H, s), 2.37 (3H, s), 2.38 (12H, s), 2.39 (12H, s), 2.41 (6H, s), 3.02-3.46 (60H, m), 7.24-7.33 (30H, m), 7.67-7.82 (30H, m).

Synthesis of Compound 23 (Figure 10)

Compound 23 (1.02 g) was obtained from Compound 11 (0.384 g, 0.399 mmol), and Compound 20 (1.20 g, 0.959 mmol) in the same manner as in the synthesis of Compound 22. Yield 77%; ^{1}H NMR ($CDCl_3$), δ: 0.88 (6H, t, J=7.0 Hz), 1.15-1.35 (36H, m), 1.43-1.56 (4H, m), 1.90-2.04 (4H, m), 2.36 (12H, s), 2.37 (3H, s), 2.38 (12H,

s), 2.39 (12H, s), 2.41 (6H, s), 3.02-3.46 (60H, m), 7.23-7.34 (30H, m), 7.67-7.82 (30H, m).

Synthesis of Compound 24 (Figure 10)

Compound 24 (0.962 g) was obtained from Compound 11 (0.387 g, 0.402 mmol), and Compound 21 (1.15 g, 0.966 mmol) in the same manner as in the synthesis of Compound 22. Yield 75%; ^{1}H NMR ($CDCl_3$), δ: 0.87 (6H, t, J=7.0 Hz), 1.15-1.35 (20H, m), 1.42-1.56 (4H, m), 1.90-2.04 (4H, m), 2.36 (12H, s), 2.37 (3H, s), 2.38 (12H, s), 2.39 (12H, s), 2.41 (6H, s), 3.02-3.46 (60H ,m), 7.23-7.34 (30H, m), 7.66-7.82 (30H, m).

Synthesis of Compound 25 (Figure 10)

To Compound 22 (0.752 g, 0.220 mmol), 25% hydrogen bromide · acetic acid solution (15 ml) was added and reacted at 100°C for 18 hours. After the solution was cooled, ethanol was added to the residue obtained by distilling the solvent and the mixture was filtered to obtain Hbr salt of Compound 25 (0.395 g) as white powder. Yield 78%; ^{1}H NMR (D_2O), δ: 0.83 (6H, t, J=7.1 Hz), 1.18-1.45 (52H, m), 1.63-1.76 (4H, m), 2.15-2.28 (4H, m), 3.07-3.16 (4H, m), 3.22-3.33 (8H, m), 3.40-3.64 (48H, m), FAB MS m/z: 1096 $[M+H]^+$.

Synthesis of Compound 26 (Figure 10)

The Hbr salt of Compound 26 (0.429 g) was obtained as a white powder from Compound 23 (0.814 g, 0.247 mmol), in the same manner as in the synthesis of Compound 25. Yield 79%; ^{1}H NMR (D_2O), δ: 0.85 (6H, t, J=7.1 Hz), 1.18-1.44 (36H, m), 1.63-1.76 (4H, m), 2.14-2.27 (4H, m), 3.08-3.16 (4H, m), 3.25-3.34 (8H, m), 3.43-3.64 (48H, m), FAB MS m/z: 984 $[M+H]^+$.

Synthesis of Compound 27 (Figure 10)

The Hbr salt of Compound 27 (0.451 g) was obtained as a white powder from Compound 24 (0.808 g, 0.254 mmol), in the same manner as in the synthesis of Compound 25. Yield 85%; ^{1}H NMR (D_2O), δ: 0.85 (6H, t, J=7.0 Hz), 1.18-1.45 (20H, m), 1.63-1.76 (4H, m), 2.14-2.29 (4H, m), 3.08-3.16 (4H, m), 3.25-3.33 (8H, m), 3.43-3.63

(48H, m). FAB MS m/z: 872 $[M+H]^+$.

Example 20: Synthesis of alkylated linear spermines

Five kinds of alkylated linear spermines shown in Figure 16 were synthesized by following the synthesis route shown in Figures 12 to 15. The following is the synthesis method.

Synthesis of Compound 28 (Figure 12)

Compound 28 (22.6g) was obtained as a white powder from spermine tetrahydrochloride (10.2 g, 29.4 mmol) by following the method of Raymond et al. (J. Med. Chem. 1988, 31, 1183-1190). Yield 94%.

Synthesis of Compound 29 (Figure 12)

Sodium hydride (60% mineral oil suspension, 0.313g, 7.81 mmol) was suspended in dimethylformamide (90 ml) and dimethylformamide solution (10 ml) of Compound 28 (5.33 g, 6.51 mmol) was dropped thereto while cooling with ice. The solution was stirred for 1 hour at room temperature, and 1-bromohexadecane (2.39 ml g, 7.81 mmol) was dropped while cooling with ice. The reaction mixture was stirred at room temperature for 6 hours, and poured into water and extracted with ethyl acetate twice. The extracts were combined and washed with distilled water and saturated brine, dried and concentrated. The residue was subjected to column chromatography with silica gel (130 g), and the fraction eluted with hexane-ethyl acetate (2:1) was collected, and Compound 29 (2.30 g) was obtained as a viscous oily substance. Yield 34%; ^{1}H NMR ($CDCl_3$), δ: 0.88 (3H, t, J=6.8 Hz), 1.13-1.35 (26H, m), 1.37-1.49 (2H, m), 1.50-1.59 (4H, m), 1.74-1.90 (4H, m), 2.36 (3H, s), 2.41 (3H, s), 2.42 (3H, s), 2.43 (3H, s), 2.96-3.16 (14H, m), 5.30 (1H, t, J=6.4 Hz), 7.24-7.34 (8H, m), 7.67-7.77 (8H, m). FAB MS m/z: 1042 $[M+H]^+$.

Synthesis of Compound 30 (Figure 12)

Compound 30 (1.73 g) was obtained as an oily substance from Compound 28 (4.06 gm 4.88 mmol) and 1-bromododecane (1.40 ml, 5.86

mmol) in the same manner as in the synthesis of Compound 29. Yield 36%; ^{1}H NMR ($CDCl_3$), δ: 0.88 (3H, t, J=7.0 Hz), 1.12-1.36 (18H, m), 1.37-1.49 (2H, m), 1.50-1.63 (4H, m), 1.72-1.90 (4H, m), 2.36 (3H, s), 2.41 (3H, s), 2.42 (3H, s), 2.43 (3H, s), 2.95-3.22 (14H, m), 5.30 (1H, t, J=6.6 Hz), 7.24-7.34 (8H, m), 7.60-7.76 (8H, m). APCI MS m/z: 987 $[M+H]^+$.

Synthesis of Compound 31 (Figure 12)

Compound 31 (1.73 g) was obtained as an oily substance from Compound 28 (4.00 gm 4.88 mmol) and 1-bromooctane (1.02ml, 5.86 mmol) in the same manner as in the synthesis of Compound 29. Yield 38%; ^{1}H NMR ($CDCl_3$), δ: 0.87 (3H, t, J=7.0 Hz), 1.13-1.36 (10H, m), 1.38-1.47 (2H, m), 1.51-1.60 (4H, m), 1.72-1.90 (4H, m), 2.35 (3H, s), 2.41 (3H, s), 2.42 (3H, s), 2.43 (3H, s), 2.95-3.22 (14H, m), 5.30 (1H, t, J=6.8 Hz), 7.24-7.35 (8H, m), 7.61-7.76 (8H, m). APCI MS m/z: 931 $[M+H]^+$.

Synthesis of Compound 32 (Figure 12)

Compound 32 (2.11 g) was obtained as an oily substance from Compound 28 (5.06 g, 6.10 mmol) and 1-bromobutane (797 μl, 7.33 mmol) in the same manner as in the synthesis of Compound 29. Yield 39%; ^{1}H NMR ($CDCl_3$), δ: 0.87 (3H, t, J=7.3 Hz), 1.16-1.34 (2H, m), 1.36-1.48 (2H, m), 1.50-1.64 (4H, m), 1.68-1.94 (4H, m), 2.35 (3H, s), 2.41 (3H, s), 2.42 (3H, s), 2.43 (3H, s), 2.90-3.22 (14H, m), 5.30 (1H, t, J=6.6 Hz), 7.20-7.38 (8H, m), 7.64-7.77 (8H, m). APCI MS m/z: 975 $[M+H]^+$.

Synthesis of Compound 33 (Figure 12)

Sodium hydride (60% mineral oil suspension, 0.582 g, 14.5 mmol) was suspended in dimethylformamide (85 ml) and dimethylformamide solution (15 ml) of Compound 28 (4.96 g, 6.06 mmol) was dropped thereto while cooling with ice. The solution was stirred for 1 hour at room temperature, and 3-[(tert-butyldimethylsilyl)oxy]-1-bromopropane (3.68 g, 14.5 mmol) was dropped while cooling with ice. The reaction mixture was stirred at room temperature for 16 hours, poured into water and extracted

with ethyl acetate twice. The extracts were combined and washed with distilled water and saturated brine, dried and concentrated. The residue was subjected to column chromatography with silica gel (160 g), the fraction eluted with hexane-ethyl acetate (3:1) was collected, and Compound 33 (5.94 g) was obtained as an oily substance. Yield 84%; ^{1}H NMR ($CDCl_3$), δ : 0.022 (12H, s), 0.84-0.91 (18H, m), 1.46-1.62 (4H, m), 1.62-1.76 (4H, m), 1.78-1.96 (4H, m), 2.41 (6H, s), 2.42 (6H, s), 3.00-3.26 (16H, m), 3.56 (4H, t, J=5.9 Hz), 7.25-7.34 (8H, m), 7.63-7.71 (8H, m). FAB MS m/z: 1185 $[M+Na]^+$.

Synthesis of Compound 34 (Figure 12)

Tetrahydrofuran solution (1M) of tetrabutylammonium fluoride (12.2 ml, 12.2 mmol) was dropped to tetrahydrofran solution (100 ml) of Compound 33 (5.94 g, 5.10 mmol) while cooling with ice. The reaction mixture was stirred at room temperature for 1 hour, diluted with water, and extracted with ethyl acetate twice. The extracts were combined and washed with distilled water and saturated brine, dried and concentrated. The obtained residue was subjected to column chromatography with silica gel (120 g), and the fraction eluted with hexane-ethyl acetate (1:4) was collected. Compound 34 was obtained as a white powder (4.55 g). Yield 95%; ^{1}H NMR ($CDCl_3$), δ: 1.54-1.68 (4H, m), 1.70-1.82 (4H, m), 1.83-1.96 (4H, m), 2.42 (6H, s), 2.43 (6H, s), 2.52 (2H, t, J=5.8 Hz, -OH), 3.06-3.26 (16H, m), 3.70 (4H, q, J=5.7 Hz), 7.25-7.35 (8H, m), 7.62-7.73 (8H, m). FAB MS m/z: 935 $[M+H]^+$.

Synthesis of Compound 35 (Figure 12)

Triphenylphosphine (6.14 g, 23.4 mmol) was added to methylene chloride solution (80 ml) of carbon tetrabromide (7.75 g, 23.4 mmol) and Compound 34 (4.55 g, 4.87 mmol)while cooling with ice. The reaction mixture was stirred for 1 hour while cooling with ice, and dried under reduced pressure. The obtained residue was subjected to column chromatography with silica gel (150 g), and the fraction eluted with 0.5% methanol-methylene chloride was collected. Compound 35 was obtained as a white

powder (4.37 g). Yield 90%; ^{1}H NMR ($CDCl_3$), δ: 1.52-1.68 (4H, m), 1.80-1.94 (4H, m), 2.04-2.18 (4H, m), 2.42 (12H, s), 3.07-3.26 (16H, m), 3.39 (4H, t, J=6.4 Hz), 7.23-7.37 (8H, m), 7.60-7.77 (8H, m). APCI MS m/z: 1059, 1061, and 1063 $[M+H]^+$.

Synthesis of Compound 36 (Figure 13)

Sodium hydride (60% mineral oil suspension, 0.209 g, 5.23 mmol) was suspended in dimethylformamide (45 ml) and dimethylformamide (8 ml) of Compound 29 (2.27 g, 2.18 mmol) was dropped thereto while cooling with ice. The solution was stirred for 1 hour at room temperature, dimethylformamide solution (7 ml) of Compound 35 (0.964 g, 0.908 mmol) was dropped while cooling with ice. The reaction mixture was stirred at room temperature for 2 hours, poured into water and extracted with ethyl acetate twice. The extracts were combined and washed with distilled water and saturated brine, dried and concentrated. The residue was dissolved in a small amount of methylene chloride, excessive amount of ethyl acetate was added thereto, and kept for 3 hours at room temperature. The deposited crystals were collected by suction filtration to obtain Compound 36 as white powder (2.04 g). Yield 75%; ^{1}H NMR ($CDCl_3$), δ: 0.88 (6H, t, J=7.0 Hz), 1.15-1.35 (52H, m), 1.37-1.47 (4H, m), 1.48-1.61 (14H, m), 1.74-1.94 (14H, m), 2.39 (36H, s), 2.95-3.22 (48H, m), 7.24-7.33 (24H, m), 7.58-7.75 (24H, m).

Synthesis of Compound 37 (Figure 13)

Compound 37 (1.09 g) was obtained as a white powder from Compound 30 (1.73 g, 1.79 mmol), and Compound 35 (0.844 g, 0.795 mmol) in the same manner as in the synthesis of Compound 36. Yield 48%; ^{1}H NMR ($CDCl_3$), δ: 0.88 (6H, t, J=7.0 Hz), 1.12-1.36 (36H, m), 1.38-1.46 (4H, m), 1.48-1.63 (14H, m), 1.74-1.92 (14H, m), 2.39 (36H, s), 2.95-3.25 (48H, m), 7.20-7.35 (24H, m), 7.55-7.75 (24H, m), FAB MS m/z: 2871 $[M+H]^+$.

Synthesis of Compound 38 (Figure 13)

Compound 38 (1.39 g) was obtained as a white powder from

Compound 31 (1.73 g, 1.86 mmol), and Compound 35 (0.820 g, 0.773 mmol) in the same manner as in the synthesis of Compound 36. Yield 65%; ^{1}H NMR ($CDCl_3$), δ: 0.87 (6H, t, J=7.0 Hz), 1.10-1.33 (20H, m), 1.38-1.47 (4H, m), 1.48-1.63 (14H, m), 1.74-1.94 (14H, m), 2.39 (36H, s), 3.00-3.15 (48H, m), 7.23-7.33 (24H, m), 7.60-7.75 (24H, m), FAB MS m/z: 2783 $[M+Na]^+$.

Synthesis of Compound 39 (Figure 13)

Compound 39 (1.33 g) was obtained as a white powder from Compound 32 (1.59 g, 1.83 mmol), and Compound 35 (0.882 g, 0.831 mmol) in the same manner as in the synthesis of Compound 36. Yield 60%; ^{1}H NMR ($CDCl_3$), δ: 0.86 (6H, t, J=7.3 Hz), 1.26-1.27 (4H, m), 1.34-1.48 (4H, m), 1.48-1.66 (14H, m), 1.74-1.94 (14H, m), 2.39 (36H, s), 2.95-3.25 (48H, m), 7.22-7.36 (24H, m), 7.55-7.75 (24H, m), FAB MS m/z: 2649 $[M+H]^+$.

Synthesis of Compound 40 (Figure 13)

To compound 36 (1.01 g, 0.339 mmol), 25% hydrogen bromide · acetic acid solution (10 ml) was added and reacted at 100°C for 6 hours. Further 25% hydrogen bromide · acetic acid solution (10 ml) was added thereto and reacted for 100°C for 19 hours. After cooling, the solvent was distilled. The obtained residue was added to ethanol and filtered to obtain a Hbr salt of Compound 40 (0.628 g). Yield 88%; ^{1}H NMR ($CDCl_3$), δ: 0.88 (6H, s), 1.20-1.45 (52H, m), 1.50-1.70 (4H, m), 1.75-1.90 (14H, m), 2.05-2.25 (14H, m), 3.00-3.30 (48H, m), APCI MS m/z: 1135 $[M+H]^+$.

Synthesis of Compound 41 (Figure 13)

Compound 41 (0.541 g) was obtained from Compound 37 (1.09 g, 0.379 mmol) in the same manner as in the synthesis of Compound 40. Yield 72%; ^{1}H NMR (D_2O), δ: 0.85 (6H, t, J=7.1 Hz), 1.18-1.43 (36H, m), 1.60-1.72 (4H, m), 1.73-1.90 (14H, m), 2.00-2.20 (14H, m), 3.00-3.33 (48H, m). APCI MS m/z: 1023 $[M+H]^+$.

Synthesis of Compound 42 (Figure 13)

Compound 42 (0.280 g) was obtained from Compound 38 (1.03

g, 0.363 mmol) in the same manner as in the synthesis of Compound 40. Yield41%; ^{1}H NMR (D_2O), δ: 0.85 (6H, t, J=7.0 Hz), 1.23-1.42 (20H, m), 1.60-1.73 (4H, m), 1.74-1.89 (14H, m), 2.00-2.22 (14H, m), 3.00-3.32 (48H, m). APCI MS m/z: 911 $[M+H]^+$.

Synthesis of Compound 43 (Figure 13)

Compound 43 (0.571 g) was obtained from Compound 39 (1.14 g, 0.430 mmol) in the same manner as in the synthesis of Compound 40. Yield 75%; ^{1}H NMR (D_2O), δ: 0.91 (6H, t, J=7.6 Hz), 1.30-1.49 (4H, m), 1.57-1.72 (4H, m), 1.73-1.90 (14H, m), 2.03-2.23 (14H, m), 3.00-3.32 (48H, m). APCI MS m/z: 799 $[M+H]^+$.

Synthesis of Compound 44 (Figure 14)

Sodium hydride (60% mineral oil suspension, 0.544 g, 13.6 mmol) was suspended in dimethylformamide (180 ml) and dimethylformamide solution (10 ml) of Compound 28 (10.1 g, 12.4 mmol) was dropped thereto while cooling with ice. The solution was stirred for 1 hour at room temperature, and 3-[(tert-butyldimetylsilyl)oxy]-1-bromopropane (3.44 g, 13.6 mmol) was dropped while cooling with ice. The reaction mixture was stirred at room temperature for 17 hours, and poured into water and extracted with ethyl acetate twice. The extracts were combined and washed with distilled water and saturated brine, dried and concentrated. The residue was subjected to column chromatography with silica gel (250 g), and the fraction eluted with hexane-ethyl acetate (1:1) was collected, and Compound 44 (4.52 g) was obtained as a white powder. Yield 37%; ^{1}H NMR ($CDCl_3$), δ: 0.017 (6H, s), 0.84-0.89 (9H, s), 1.48-1.58 (4H, m), 1.60-1.71 (2H, m), 1.72-1.93 (4H, m), 2.41 (4H, s), 2.42 (4H, s), 2.43 (3H, s), 2.93-3.25 (14H, m), 3.55 (2H, t, J=6.0 Hz), 5.30 (1H, t, J=6.4 Hz), 7.23-7.34 (8H, m), 7.55-7.77 (8H, m). APCI MS m/z: 991 $[M+H]^+$.

Synthesis of Compound 45 (Figure 14)

Sodium hydride (60% mineral oil suspension, 0.0499 g, 1.24 mmol) was suspended in dimethylformamide (20 ml) and dimethylformamide solution (5 ml) of Compound 44 (1.02 g, 1.04

mmol) was dropped thereto while cooling with ice. The solution was stirred for 1 hour at room temperature, and dimethylformamide (5 ml) of Compound 35 (0.506 g, 0.471 mmol) was dropped thereto while cooling with ice. The reaction mixture was stirred at room temperature for 22 hours, poured into water and extracted by ethyl acetate twice. The extracts were combined and washed with distilled water and saturated brine, dried and concentrated. The residue was subjected to column chromatography with silica gel (30 g), and the residual Compound 44 was eluted with hexane-ethyl acetate (1:1). The fraction eluted with 5% methanol-metylene chloride was then collected to obtain Compound 45 (0.92 g) as a white powder. Yield 68%; ^{1}H NMR ($CDCl_3$), δ : 0.013 (12H, s), 0.85-0.88 (18H, s), 1.45-1.58 (14H, m), 1.60-1.74 (4H, m), 1.76-1.94 (14H, m), 2.30-2.48 (36H, m), 2.95-3.25 (48H, m), 3.54 (4H, t, J=6.0 Hz), 7.23-7.34 (24H, m), 7.58-7.73 (24H, m).

Synthesis of Compound 46 (Figure 14)

Tetrahydrofuran solution (1 M) of tetrabutylammonium fluoride (0.75 ml, 0.75 mmol) was dropped to tetrahydrofran solution (18 ml) of Compound 45 (0.901 g, 0.313 mmol) while cooling with ice. The reaction mixture was stirred at room temperature for 1 hour, diluted with water, and extracted with methylene chloride twice. The extracts were combined and washed with distilled water and saturated brine, dried and concentrated. The obtained residue was subjected to column chromatography with silica gel (17 g), and the fraction eluted with 2% methanol-methylene chloride was collected to obtain Compound 46 as a white powder (0.780g). Yield 94%; ^{1}H NMR ($CDCl_3$), δ : 1.46-1.60 (14H, m), 1.68-1.76 (4H, m), 1.78-1.94 (14H, m), 2.40 (36H, s), 2.47 (2H, t, J=6.0 Hz, -OH), 2.98-3.24 (48H, m), 3.67 (4H, q, J=5.9 Hz), 7.24-7.33 (24H, m), 7.55-7.75 (24H, m).

Synthesis of Compound 47 (Figure 15)

Carbon tertabromide (0.947 g, 2.86 mol) and triphenylphosphine (0.750 g, 2.86 mmol) were added to methylene chloride solution (12 ml) of Compound 46 (0.761 g, 0.286 mmol) while cooling

with ice. The reaction mixture was stirred for 1.5 hour at room temperature and dried under reduced pressure. The obtained residue was subjected to column chromatography with silica gel (40 g), and the fraction eluted with hexane-ethyl acetate (1:3) was collected. Compound 47 was obtained as a white powder (0.744 g). Yield 94%; ^{1}H NMR ($CDCl_3$), δ: 1.40-1.66 (14H, m), 1.74-1.97 (14H, m), 2.02-2.16 (4H, m), 2.40 (36H, s), 2.94-3.24 (48H, m), 3.37 (4H, t, J=6.4 Hz), 7.23-7.35 (8H, m), 7.55-7.77 (24H, m).

Synthesis of Compound 48 (Figure 15)

Sodium hydride (60% mineral oil suspension, 0.0307 g, 0.769 mmol) was suspended in dimethylformamide (18 ml) and dimethylformamide solution (5 ml) of Compound 29 (0.668g, 0.641 mmol) was dropped thereto while cooling with ice. The solution was stirred for 1 hour at room temperature, and dimethylformamide solution (5 ml) of Compound 47 (0.731 g, 0.263 mmol) was dropped while cooling with ice. The reaction mixture was stirred at room temperature for 4 hours, poured into water and extracted with ethylene chloride twice. The extracts were combined and washed with distilled water and saturated brine, dried and concentrated. The residue was subjected to column chromatography with silica gel (30 g), the residual Compound 29 was eluted with hexane-ethyl acetate (1:1), and the fraction eluted with methanol-methylene chloride was collected to obtain Compound 48 (0.761 g) as white powder. Yield 62%; ^{1}H NMR ($CDCl_3$), δ: 0.88 (6H, t, J=6.4 Hz), 1.12-1.34 (52H, m), 1.40-1.48 (4H, m), 1.49-1.62 (24H, m), 1.73-1.95 (4H, m), 2.38 (30H, s), 2.39 (30H, s), 2.97-3.27 (80H, m) 7.23-7.33 (40H, m), 7.55-7.74 (40H, m).

Synthesis of Compound 49 (Figure 15)

25% Hydrogen bromide · acetic acid solution (15 ml) was added to compound 48 (0.742 g, 0.158 mmol), and the mixture was reacted at 100°C for 4 hours, Further 25% hydrogen bromide · acetic acid solution (7.5 ml) was added thereto, and the mixture was reacted for 100°C for 19 hours. After cooling, the solvent was distilled and the obtained residue was added to ether, and filtered to obtain

a Hbr salt of Compound 49 (0.442 g). Yield 86%; ^{1}H NMR (D_2O), δ: 0.93 (6H, m), 1.30-1.60 (52H, m), 1.70-1.80 (4H, m), 1.80-2.00 (24H, m), 2.10-2.35 (24H, m), 3.08-3.48 (80H, m), APCI MS m/z: 1619 $[M+H]^+$.

Example 21: Preparation of stock solution

The Hbr salt of the 8 kinds of the derivatives synthesized in Examples 19 and 20 were weighed, sterilized water was added, and the pH was adjusted to around 7 with 1N-NaOH to prepare the various stock solutions. Compounds sparingly soluble in water were heated at 80°C for 15 to 30 min, and cooled to room temperature. pH was adjusted and the solution was treated by a probe-type ulrasonicator (BRONSON, 20 Dutycycle, for 5 min). All carrier solutions were filtered with a filter (DISMIC-25cs, Cellulose acetate, 0.2 μm, ADVANTEC TOYO). The concentrations of the carrier solution were expressed as a molar concentration of amine nitrogen. Namely, the stock solution was 10 mM mol number of the positive charges (10 nmol/μl). These stock solutions were kept at 4°C until used. At the time of use, the precipitated stock solutions were dissolved by heating at 80°C for 15 to 30.

Example 22: *In vitro* gene introduction of alkylated linear ethylenimines

The gene expression using alkylated linear ethyleneimine in human liver carcinoma (Hep G2) was examined (Table 3). In the table, the numerical values indicate mean $\pm$ SD (n=3). The compounds TEL-D8, TEL-D12, TEL-D16 were used and the method in Examples 12 to 16 followed. Among them, TEL-D16 showed the highest gene expression. The gene expression in TEL-D16 was about 10 times higher than LipofectAMINE in the presence or absence of serum addition at transfection.

Table 3

Compound Name	Compound No.	Charge ratio (eq)	Gene expression LacZ (RLU/μg·sec)	
			No serum	50% serum
TEL-D8	27	9	179±41	163±23
		18	177±67	146±20
TEL-D12	26	9	295±198	134±10
		18	4,130±3,316	128±6
TEL-D16	25	9	106,494±22,206	23,608±8,655
		18	155,013±8,191	17,593±6,292
LipofectAMINE			9,345±9,573	2,373±1,620

Example 23: *In vitro* gene introduction of alkylated linear spermines

The gene expression using alkylated linear spermines in human liver carcinoma (Hep G2) was examined (Table 4). In the table, the numerical values indicate mean ± SD (n=3). The compounds TSL-D4, TSL-D8, TSL-D12, TSL-D16 and PSL-D16 were used and the method in Examples 12 to 16 followed.

Table 4 shows the result. The gene expression in TSL-D8 and TSL-D16 was about 10 times higher and in PSL-D16 was about 5 time higher than LipofectAMINE, in the case of not adding serum at transfection. While in addition of serum, the gene expression in TSL-D16 was 10 times of that in LipofectAMINE or higher.

Table 4

Compound Name	Compound No.	Charge ratio (eq)	Gene expression LacZ (RLU/μg·sec)	
			No serum	50% serum
TSL-D4	43	9	140±14	142±5
		18	479±570	2,517±2,361
TSL-D8	42	9	163,488±55,392	269±5
		18	141,151 ± 111,159	291±9
TSL-D12	41	9	1,999±2,270	144±3
		18	10±14	132±8
TSL-D16	40	9	142,963±26,041	50,683±8,892
		18	17,435±12,412	23,393±10,319
PSL-D16	49	9	63,231±16,415	6,721±2,608
		18	563±412	2,702±383
LipofectAMINE			9,345±9,573	2,373±1,620

Example 24: Preparation of human pancreatic adenocarcinoma and human glioblastoma

Human pancreatic adenocarcinoma BxPc-3 (Primary pancreatic adenocarcinoma, from Dainippon Chemicals) was cultured in RPMI medium (GIBCO BRL) with 10% FBS (GIBCO BRL) at 37°C in the CO_2 incubator. In the same manner, human glioblastoma SK-N-MC (Glioblastoma, from ATCC) was cultured in D-MEM medium with 10% FBS at 37°C in the CO_2 incubator. Each cell was treated with tripsin, washed with each medium cooled with ice three times, and adjusted to 1 x 10^8 cells/ml. The cells were subcutaneously transplanted to the mice within 40 min after the preparation.

Example 25: Preparation of tumor-bearing mice into which tumor-cells were subcutaneously transplanted

Each cancer cell prepared in Example 24 (0.2 ml each) was administered (injection needle 29Gx1/2", TERMO) to two sites in the right and left of abdomen and two sites of the upper and lower back (four sites in total) of SCID mouse (male, 5-week old, Nippon Clea). Two weeks after the administration, the neoplastic diameter of human pancreatic adenocarcinoma BxPc-3 was 0.5 to 0.8

cm in height and 0.5 to 0.8 cm in width. On the other hand, the neoplastic diameter of human glioblastoma SK-N-MC was 0.9 to 1.4 cm in height and 1.1 to 1.75 cm in width.

Example 26: Method for preparing plasmid

Plasmid pCAG-EGFP was constructed by inserting GFP of green protein from jelly fish (Clontech, pEGFP-N1) into plasmid pCAGGS. The stock solution of this plasmid (8 mgDNA/ml) was diluted with 5% glucose (Fuso Pharmaceutical Industries, glucose injection solution) to prepare the 500 μg DNA/ml DNA solution. The mol number of the negative charges in 1μg DNA was 3 nmol, and the mol number of the negative charges in 500μg DNA was 500μg DNA/ml x 3 nmol = 1,500 nmol.

Example 27: Method for preparing each derivative carrier solution

The stock solution of Example 21 was prepared with 5% glucose so that N/P (N: the mol number of the positive charges of nitrogen in carrier/P: the mol number of the negative charges of phosphate in DNA) was 0.3 equivalent.

Example 28: Method for forming a DNA complex

The DNA solution of Example 26 (100 μl) was transferred to a 1.5-ml Eppendorf tube. The carrier solution of Example 27 (100 μl) was added thereto and gently vortexed. The mixture was kept at room temperature for 30 min or longer to form the DNA complex (N/P = 0.3 equivalent). The complex was kept at 4°C overnight and used for the following experiment.

Example 29: Gene expression in the tumor-bearing mouse

The DNA complex of Example 28 (50 μl, 12.5 μg DNA) was directly administered into the tumor of the tumor-bearing mouse, prepared in Example 25 (injection needle 29Gx1/2", TERMO). Ten days after the administration of the DNA complex, the mouse was killed by dislocation of cervical vertebrae under the anesthesia with ether, and the rooted tumor was excised with scissors from the subcutis. The expression of GFP gene was examined by excising

the tumor and observing the internal neoplastic tissue under the fluorescence microscope. Table 5 shows the result. In the table, a GFP expression intensity of (+), (±), and (-) indicate the strong, weak, and no expression, respectively. In human pancreatic adenocarcinoma BxPc-3, TSL-D8, TSL-D16, PSL-D16, TEL-D8 TEL-D16 showed the fluorescence intensities stronger than naked DNA. Similarly, in human glioblastoma SK-N-MC, TEL-D8 showed GFP fluorescence stronger than naked DNA.

Table 5

Compound	Compound No.	Gene expression in subcutaneous tumor in the mouse	
		BxPc-3	SK-N-MC
TEL-D8	27	++	±
TEL-D12	26	-	-
TEL-D16	25	++	±
TSL-D4	43	±	±
TSL-D8	42	++	++
TSL-D12	41	-	-
TSL-D16	40	++	±
PSL-D16	49	++	±
Naked DNA		±	- / ±

Industrial Applicability

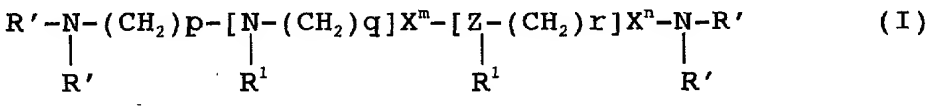
The present invention provides a composition comprising as an ingredient, a polyalkylenimine into which multiple hydrophobic groups have been introduced, and a method for introducing genes using said composition. Thus, the present invention enables a more effective transport of negatively charged physiologically active substances to cells when compared to using conventional cationic macromolecules.

CLAIMS

1. A composition comprising a polyealkylenimine having two or more hydrophobic groups or its salt.

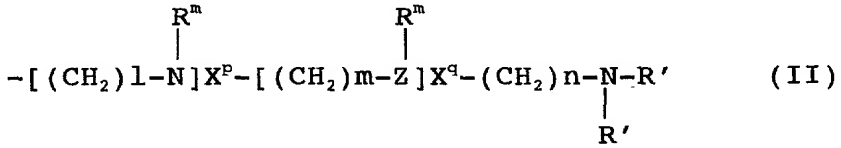
2. The composition of Claim 1, wherein the hydrophobic group is a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid residue.

3. The composition of Claim 1, wherein the polyalkylenimine having two or more hydrophobic groups is a compound represented by formula (I):



(I)

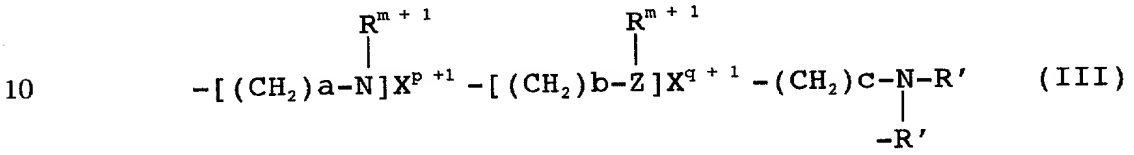
wherein the base skeleton may contain an amide bond; Z represents a carbon or nitrogen atom; R′ represents hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid residue; two R′s binding to the same nitrogen atom can be identical or different; a side chain R_1 is hydrogen, a cholesterol residue, saturated or unsaturated alkyl group, saturated or unsaturated acyl group, or saturated or unsaturated acyloxycarbonyl group, phospholipid residue, or below formula (II); and p, q, r, X^n, X^m represent arbitrary natural numbers:



(II)

wherein the base skeleton and the side chain R^m may contain an amide bond; Z represents a carbon or nitrogen atom; R′ represents hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid residue; two

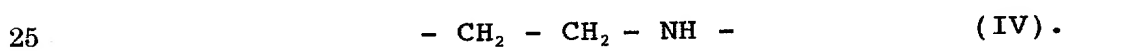
R's binding to the same nitrogen atom can be identical or different; R^m is hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, or a saturated or unsaturated acyloxycarbonyl group, a phospholipid residue, or below formula (III); and l, m, n, X^p, X^q represent arbitrary natural numbers:



(III)

wherein the base skeleton and the base skeleton of the side chain R^{m+1} may contain an amide bond; Z represents a carbon or nitrogen atom; R' represents hydrogen, a cholesterol residue, a saturated or unsaturated alkyl group, a saturated or unsaturated acyl group, a saturated or unsaturated acyloxycarbonyl group, or a phospholipid residue; two R's binding to the same nitrogen atom can be identical or different; and a, b, c, X^{p+1}, X^{q+1} represent arbitrary natural numbers.

4. The composition of any one of Claims 1 to 3, comprising the repeating structure of formula (IV) in the base skeleton:



(IV).

5. The compositions of Claim 4, wherein two to five molecules of tetraethylenepentamine are linked in a linear manner.

6. The composition of Claim 5, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups.

7. The composition of Claim 5, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of a butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl,

heptadecyl, and octadecyl groups.

8. The composition of Claim 4, wherein the structure containing the formula (IV) are linked in a branched manner.

9. The composition of Claim 8, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups.

10. The composition of Claim 8, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl groups.

11. The composition of any one of Claims 1 to 3, wherein the base skeleton contains a spermine structure.

12. The composition of Claim 11, wherein two to five molecules of spermines are linked in a linear manner.

13. The composition of Claim 11, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups.

14. The composition of Claim 11, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of a butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl groups.

15. The composition of Claim 11, wherein the spermine structure is linked in a branched manner.

16. The composition of Claim 14, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group consisting of ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, and eicocyl groups.

17. The composition of Claim 14, wherein any two or more of side chains R', R^1, R^m, or R^{m+1} comprise a group selected from the group

consisting of butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl groups.

18. The composition of any one of Claims 1 to 17, further comprising phospholipid.

19. The composition of Claim 18, wherein the phospholipid is neutral or basic phospholipid.

20. The composition of Claim 19, wherein the phospholipid comprises phosphatidylethanolamine, or phosphatidylcholine skeleton.

21. The composition of Claim 20, wherein the phospholipid is dioleylphosphotidylethanolamine, or phosphotidylcholine.

22. A complex comprising a physiologically active substance comprising a negative charge and a composition of any one of Claims 1 to 21.

23. The complex of Claim 22, wherein the physiologically active substance comprising a negative charge is a nucleic acid or its derivative.

24. A method for introducing a physiologically active substance comprising a negative charge to cells, said method comprising a step of contacting the complex of Claim 22 or 23 with cells.

25. A kit for preparing the composition of any one of Claims 19 to 21, comprising phospholipid and a polyalkylenimine having two or more hydrophobic groups per molecule or its salt.